FURTHER OBSERVATIONS ON AN EXPERIMENTALLY PRODUCED SARCOMA OF THE CHICKEN.*

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In a series of publications from The Rockefeller Institute appearing during the period from 1910 to 1914, five transplanted chicken tumors were described, all of which proved transmissible by cell-free filtrates or desiccates of the tumor material.[1] More recently several other transplantable chicken tumors have been reported and from all so far studied it has been possible to separate an agent from the cells capable of reproducing the tumors.

Murphy and Landsteiner,[2] in the hope of gaining some information on the nature of the causative agents of this chicken tumor group, succeeded in producing typical sarcomas by the combined injection of tar and embryonic tissue in adult hens. One of these was transplantable but all attempts to transmit it by filtrates or desiccates failed in the early generations. As this tumor remained the only transplantable chicken sarcoma which could not be transmitted by an agent separable from the cells, it was considered worth while to continue the attempts under varying conditions on the later generations. In the 3 years since the original publication, the neoplasm has been repeatedly transplanted and continues to grow quite readily. In the present report we have brought together the results of all the

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[1] Rous, P., *J. Exp. Med.*, 1911, xiii, 397. Rous, P., Murphy, Jas. B., and Tytler, W. H., *J. Am. Med. Assn.*, 1912, lix, 1793. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52. Rous, P., *J. Exp. Med.*, 1914, xix, 570. Lange, L. B., *J. Exp. Med.*, 1914, xix, 577.

[2] Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 6, 807.

numerous attempts to disassociate an agent from the cells of this tumor, which for convenience is called Chicken Tumor 9.

Filtration Experiments.

The same general methods of filtration which gave positive results with the other chicken tumors, were used in the following experiments.

Filtration Method.—About 25 gm. of fresh tumor tissue, previously trimmed of all necrotic and muscle tissues, was finely chopped in a meat grinder and thoroughly ground in a mortar with sterile sand. To the suspension was added about 400 cc. of Ringer's solution and the entire mixture was thoroughly shaken for 20 minutes. It was then centrifuged for 15 minutes to remove the sand and solid portions of tissue, and the supernatant fluid was decanted. To it a suspension of a 24 hour culture of *B. prodigiosus* was added as a means of testing the permeability of the filter and the fluid was passed through a Berkefeld V candle. A trace of kieselguhr was added to the fresh filtrate prior to injecting. Injections of varying amounts (5 to 20 cc.) of filtrate were made into the breast muscles of normal adult chickens.

TABLE I.

Experiment No.	Size filter	Tumor generation	No. of fowls	No. of regions injected	Growths +	Growths −
1	V and N	1st A	8	16	0	16
2	V and N	1st A	10	20	0	20
3	V	2nd A	8	16	0	16
4	V	2nd C	10	20	0	20
5	V	3rd B	10	20	0	20
6	V	5th A	6	12	0	12
7	V	7th A	8	16	0	16
8	V	13th B	5	10	0	10
9	V	17th D	5	10	0	10
10	V	21st F	2	4	0	4
Msl.	V		25	48	0	48
			97	192	0	192

The above table shows the condensed results of the experiments in which Berkefeld filtrate of Chicken Tumor 9 was injected into normal adult chickens. The Msl. fowls are from later experiments in which filtrate alone was injected as control in tests when filtrate was injected with other substances.

TABLE II.

Experiment No.	Age of tumors	Size of tumor		"Takes" in tumor generation
		Right	Left	
	wks.	*cm.*	*cm.*	*per cent*
1	6	5.0 × 2.3	4.9 × 3.2	10
2	7	4.2 × 2.7	4.2 × 3.1	66.6
3	10	6.0 × 5.2	5.7 × 4.6	10
4	6	6.3 × 4.7		75
5	3	6.4 × 4.3	7.0 × 4.5	80
6	6	6.0 × 3.5	6.6 × 4.5	66.6
7	5	6.0 × 4.2	4.0 × 3.2	80
8	3	4.0 × 5.8	7.2 × 3.5	62.5
9	5	6.5 × 4.5	7.0 × 4.9	50
10	7	10.0 × 5.0	10.0 × 5.0	75

The above table gives the data in regard to the tumors used in the filtration experiments summarized in Table I. It gives the size of the tumors used, the length of time since inoculation and the number of tumor "takes" in the same generation.

The filtration test in this group of ten experiments (Table I) was made with ten different tumors obtained from the first to the twenty-first generations. Ninety-seven chickens received the filtrates including those of the miscellaneous group made up of controls from other experiments. The fowls were injected generally in both breasts and kept under observation from 3 to 6 months. Not a single positive result was obtained. That this failure is not due to lack of malignancy of the tumor may be judged by the growth rate and percentage of "takes" on transplantation as shown in Table II. At the time most of the tests were made this tumor was growing at a rate quite equal to that of several of the other transplantable chicken tumors which were easily transmissible by filtrates.

The Injection of the Filtrate into Growing Embryomas.

The tumor originally developed in an embryoma and the possibility that the young elements in an actively growing tissue would create a more suitable environment for its successful transmission by filtrate led us to undertake the following experiments.

Experiment.—Hashed 7 day old chick embryos were injected into both breast muscles of five adult chickens. On the 12th day following the injection, at the time when the embryonic tissue was actively growing, 10 cc. of freshly prepared tumor filtrate was injected into and around the growing embryoma. Another group of five chickens was injected into each breast muscle with 10 cc. of a mixture composed of 12 cc. of hashed 7 day old chick embryos and 90 cc. of fresh tumor filtrate; while a control group of five chickens were injected with the Berkefeld filtrate of the tumor alone.

Several embryomas continued to grow actively for a short time after the filtrate injection but microscopic sections, prepared from pieces removed at operation, showed them to be composed of the cartilage and bone usually found in typical embryoma, without any indication of malignant transformation. These nodules eventually retrogressed and finally disappeared entirely. No evidence of tumor growth was observed in any of the fifteen chickens employed in this experiment.

Injection of the Filtrate into the Developing Embryo.

It was observed by Murphy and Rous[3] that the Berkefeld filtrate of Chicken Tumor 1 rapidly gave rise to tumor nodules when the filtrate was injected into developing chick embryos. It was thought that a tumor might possibly result from the injection into the relatively unresistant chick embryo[4] of the filtrate of Chicken Tumor 9.

Experiment.—A small rectangular piece was cut from the shell of a fertile egg by means of a shortened cataract knife. Exceptional precautions are necessary to avoid cutting through the shell membrane. With a pair of sterile forceps this membrane next was torn aside exposing the chick. A syringe of 2 cc. capacity fitted with a 1 inch, 20 gauge needle was filled with freshly prepared Berkefeld filtrate of the tumor tissue, and 1 cc. injected into the embryonic membranes. The small piece of shell was carefully replaced and the edges sealed with paraffin. The age of the embryos at the time of inoculation varied from 7 to 10 days. After inoculating, the eggs were returned to the incubator until the 19th day, when they were opened for examination. Thirty-three embryos so examined in our experiments failed to reveal any evidence of tumor nodules.

The Addition of a Mucoid Fluid from a Filtrable Chicken Tumor.

The following experiment was planned with the possibility in mind that the mucoid fluid, notably present in the tissue of some of the

[3] Murphy, Jas. B., and Rous, P., *J. Exp. Med.*, 1912, xv, 119.
[4] Murphy, Jas. B., *J. Exp. Med.*, 1913, xvii, 482.

filtrable chicken tumors, might have qualities of rendering the filter permeable to the causative agent of the tar tumor; for it is known that other factors beside the porosity of the filter influence the result of filtration.

Experiment.—The mucoid fluid was aspirated from a large Chicken Tumor 1 and filtered through filter paper to remove any lumps of tissue. It was then sealed in a glass tube and immersed in a water bath for 30 minutes, the temperature of which was kept at 55°C. in order to kill any tumor cells present and to render inactive the tumor-producing agent. Thirty cc. of this fluid was added to 30 gm. of finely chopped tar tumor tissue and ground with sterile sand in a mortar. The remainder of the filtering process was carried out in a manner similar to that of the previous experiments.

Six adult chickens were injected into each breast muscle with 10 cc. of this filtrate and as controls two chickens were injected with 10 cc. of the inactivated mucoid fluid. Another control group of four chickens were injected with 10 cc. of filtrate freshly prepared from the tumor tissue alone. All of the fowls remained negative for tumor growth during 2 months of observation.

Experiments with Desiccated Material from the Tar Sarcoma 9.

The failure to obtain any positive results by filtration led us to attempt a series of experiments in which desiccated Chicken Tumor 9 tissue was used in place of the filtrate. If this tumor was found resistant to drying, a partial analogy to the previously described transplantable chicken tumors could probably be established.

Experiments.—Large, actively growing tumors were removed under aseptic precautions, trimmed of all adhering muscle and necrotic tissue and ground in a meat grinder. The mashed tissue was then evenly spread over the bottom of a glass dish and placed in a desiccating jar containing a layer of sulfuric acid. The jar was evacuated to 4 mm. pressure and immediately placed in a freezing box where the temperature was several degrees below 0°C. In 3 to 4 days, or when the tissue was thoroughly dry, the scaly substance was pulverized in a mortar and about 2 gm. of this material was emulsified in 20 cc. of either sterile distilled water or Ringer's solution. From 2 to 5 cc. of this emulsion was injected into the breast muscles of normal chickens.

Five experiments were conducted with the desiccated material obtained from tumors in the first, second, third and sixth generations and in all forty-two chickens were injected into eighty-four regions. The fowls were kept under observation for from 3 to 6 months. No tumors developed. A summary is given in Table III.

TABLE III.

Summary of the Desiccation Experiments.

Experiment No.	Tumor generation	Size of tumor	Age of tumor	No. of chickens injected	No. of regions injected	Results +	Results −
		cm.	*wks.*				
1	1st A	5.0 × 3.3 4.9 × 3.2	6	11	22	0	22
2	1st F	4.2 × 3.6 3.0 × 2.4	6	5	10	0	10
3	2nd C	4.2 × 2.6 4.2 × 3.1	7	10	20	0	20
4	3rd B	6.3 × 4.7	6	11	22	0	22
5	6th B	6.0 × 3.5 6.6 × 4.5	6	5	10	0	10
5				42	84	0	84

The Addition of Embryonic Tissue to the Desiccated Tumor Tissue.

As the original tumor was obtained by the injection of embryonic tissue and tar, as noted above, it seemed possible that the addition of some fresh, living embryonic tissue, to an emulsion of the desiccated tumor material, might produce the necessary stimulus for a positive growth.

Experiment.—A mixture was prepared consisting of equal portions of 7 day old chick embryonic tissue, and desiccated tumor tissue emulsified in Ringer's solution. Two cc. of this combination was injected into thirteen normal hens, and weekly observations were recorded for several months. As in all of the previous experiments, these animals remained negative, without suggestion of tumor formation.

Inoculation of the Developing Embryo with Desiccated Tumor Tissue.

In a series of eight experiments we injected into the chick embryo small portions of freshly prepared desiccate of tumors from the sixth to the sixteenth generations. Out of ninty-three living embryos injected between the 6th and 8th days and examined on the 18th day

of incubation, not one had developed any suggestion of tumor-like nodules.

From the results of the foregoing experiments it seems certain that Chicken Sarcoma 9 cannot be propagated from the cell-free filtrate of the tumor or the desiccated tumor tissue by any of the usual methods.

Further Attempts to Transmit Chicken Tumor 9 by the Addition of "Cultures" of This Tumor and Normal Tissues to Filtrates.

Gye[5] has shown that it is possible to obtain growths of Chicken Tumor 1, after the filtrate of this tumor has been inactivated by chloroform, providing there is added to the filtrate an equal amount of fluid obtained from "cultures" of malignant tissue. More recently Murphy[6] and Flu[7] have demonstrated that not only malignant tissue but normal tissue "cultures" as well, will bring about this reactivation. With the idea that some essential factor might be removed by filtration or destroyed by drying Chicken Tumor 9, or that the agent is naturally feeble, we have attempted to supply the factor or augment the activity of this agent by the substances which activate the chloroform filtrate of Chicken Tumor 1.

Experiments.—The base of the medium used throughout these experiments was Hartley's broth to which had been added .2 per cent KCl, .7 per cent dextrose and 1 cc. of fresh rabbit serum. Pieces of tumor or embryonic tissue were introduced and the "cultures" were incubated under strict anaerobic conditions at 37.5°C.

To portions of freshly prepared Berkefeld filtrate of Chicken Tumor 9 were added equal amounts of supernatant fluid obtained from 3 day anaerobic "cultures" of rat placenta and chicken embryos. Five cc. of each of these combinations was injected into two groups of four normal hens, the experiment being controlled by injecting 10 cc. of the chicken tumor extract alone into two normal chickens. The animals were observed for a period of 2 months after which they were discarded as no tumors had developed.

In a second experiment, we mixed together equal portions of Chicken Tumor 9 filtrate and the supernatant fluid from 5 day "cultures" of the same tumor. Ten cc. of this mixture was injected into both breasts of three adult chickens. Another group of chickens were injected with 20 cc. of the extract alone to serve as controls. Not a single tumor developed from any of these injections.

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[5] Gye, W. E., *Lancet*, 1925, ii, 109.

[6] Murphy, Jas. B., *J. Am. Med. Assn.*, 1926, lxxxvi, 1270.

[7] Flu, P. C., *Centr. Bakt., 1. Abt., Orig.*, 1926, cix, 332.

In a third experiment we attempted to activate the Chicken Tumor 9 filtrate by adding to it 7 day "cultures" of Chicken Tumor 1, but without results.

TABLE IV.

Materials	No. of fowls	No. of injections	Results Positive	Results Negative
Filtrate alone in adult	97	192	0	192
Filtrate in growing embryoma	10	20	0	20
Filtrate in developing embryo	33	33	0	33
Filtrate and mucoid fluid from C. T. 1	6	12	0	12
Desiccate alone in adult	42	84	0	84
Desiccate and embryo tissue	13	26	0	26
Desiccate in developing embryo	93	93	0	93
Filtrate and "culture" fluid	13	13	0	13
Total	307	473	0	473

A summary of the various experiments with filtration and desiccation of Chicken Tumor 9 is given in Table IV. There is no indication that a substance exists separable from the cells by these methods, capable of reproducing the tumor.

Attempts to Demonstrate a Diffusible Substance from "Cultures" of Chicken Tumor 9.

While attempting to discover some method by which the hypothetical agent of the tar tumor could be separated from the cells, it was observed that the fluid from the "cultures" of this tumor sometimes produced tumors when the cultivation was made in sterile Ringer's solution and the tubes allowed to stand in the ice chest under anaerobic conditions for a period of 5 days or longer. These observations indicated the possibility that an active substance had diffused from the tumor fragments. In order to test the matter, the following experiments were planned.

Experiment.—1. To the filtrate of Chicken Tumor 9 was added an equal amount of supernatant fluid from "cultures" in Ringer's solution of Chicken Tumor 9 which had been kept in the ice chest for 5 days under strict anaerobic conditions. Two chickens were injected with 10 cc. of this mixture. One of these chickens

subsequently developed a tumor typical of Chicken Tumor 9. However, it was observed that one of the two control chickens, previously injected with 5 cc. of "cultural" fluid alone had also developed a fair sized nodule which eventually grew extensively and resembled Chicken Tumor 9.

Experiment.—2. A large number of "cultures" of Chicken Tumor 9 tissue in both Hartley's medium and Ringer's solution were prepared. The "cultures" were anaerobically sealed and placed in the ice chest for a period of 6 days. One half of each group of "cultures" were united and filtered through a Berkefeld V filter, while the fluid from the other half was decanted and centrifuged several times at high speed. Both the filtrate and the centrifuged cultures were injected into individual groups of two normal chickens each in measured amounts of 5 cc. No tumors developed in chickens injected with the filtered "cultures" from Hartley's medium, the supernatant fluid from Hartley's medium or from the injection of the filtered Ringer's solution "cultures." However, a typical Chicken Tumor 9 was observed in one of the two chickens injected with the centrifuged supernatant fluid from the Ringer's solution "cultures."

Experiment.—3. The general procedure of this experiment was identical with that of the preceding experiment. Here again it was observed that all three of the chickens injected with the filtrate were negative for tumor growth, whereas both of those injected with supernatant fluid from the Ringer's solution "cultures" developed tumors.

Experiment.—4. In this experiment we substituted sterile distilled water for Ringer's solution in one set of tubes while in another Hartley's medium was used. A long period of observation of the ten fowls used failed to show any tumors resulting from the injections.

As the filtrates of these "cultures" always failed to give tumors it was concluded that the occasional tumors resulting from the injection of the centrifuged material were due to the presence of living cells. This supposition was strengthened by the fact that the sediment contained large numbers of unquestionably living cells. The result then cannot be considered as giving evidence of the presence of an agent separable from the tumor tissue.

DISCUSSION.

The experiments reported here represent an extension of the original study of a tar tumor reported by Murphy and Landsteiner. In its general features the growth is a typical neoplasm with minor histological differences from other chicken tumors studied but it differs no more from these tumors than the individual tumors in the group differ from each other. Yet it appears to differ from all other transplant-

able chicken tumors having for their origin a spontaneous growth, in that despite many efforts no causative agent has been separated from the living cell. It is, of course, possible that some new method or change in technique may lead to a positive result, yet considering the very wide range of conditions resorted to in this study, its negative result would appear significant. The possibility that the agent might be highly susceptible to oxidation has not been completely tested but the negative results obtained in this laboratory with extracts of rat and mouse tumors filtered under anaerobic conditions indicate that this possibility is not of importance in explaining the failure in filtrability. That the agent might require contact with cells of the type from which the tumor presumably arose has been well covered by injecting the filtrate and desiccate into growing embryoma. That the failure is not due to natural resistance in the chicken is shown by the fact that the developing embryo, an organism without resistance,[4] failed to yield growths on the injection of filtrate or desiccate.

While Chicken Tumor 9 is not so rapid in its growth as Chicken Tumor 1, yet it is more rapid than several of the other transplantable spontaneous tumors[8] which have been easily transmitted by filtrates. It would seem, therefore, that the failure of filtrability in its case is not explainable on the basis of lack of malignancy.

For the present this tumor must stand as an exception in the chicken tumor group, in that it resembles the mammalian tumors in the failure to be transmitted by an agent separable from the living cell.

SUMMARY.

Numerous attempts have been made by us to separate from the cells of a tar sarcoma of the chicken (Chicken Tumor 9) a causative agent for the growth. Experiments with filtrates and desiccates injected as such or in combinations with embryonic tissues have all failed to give positive results. So too have injections of filtrates and desiccates into developing chick embryos failed to yield a response. The results confirm those of previous work with the tumor in this laboratory. The growth would appear to differ in a fundamental respect from all tumors of the fowl previously studied.

[8] Tytler, W. H., *J. Exp. Med.*, 1913, xvii, 466. Rous, P., and Lange, L. B., *J. Exp. Med.*, 1913, xviii, 651. Rous, P., *J. Exp. Med.*, 1914, xix, 570.

THE USE OF HISTAMINE AS A STANDARD TEST FOR DIMINISHED RESISTANCE IN SUPRARENALECTOMIZED RATS.

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The resistance of suprarenalectomized animals to a number of nonspecific poisons is markedly reduced, as has been shown by several independent workers (1–6). These observers employing such poisons as cobra venom, curare, morphine, diphtheria toxin and typhoid vaccine were able to demonstrate a striking difference in resistance between normal and suprarenalectomized rats. In comparing the results obtained in different laboratories with the same poison, considerable differences in the lethal dose appeared to exist. With some of the poisons, as for example, typhoid vaccine, it seemed likely that the difference could be explained by a lack of uniformity of the preparation. Inasmuch as this test may have considerable value in future experimental work, it seemed advisable to secure additional data concerning certain of these poisons in order to find a stable substance having a relatively constant ratio between the lethal dose for suprarenalectomized rats and that for normal rats. This ratio should be at least 1:20. If such a substance could be found, the test might be standardized. In our attempts to standardize this test, we have used, up to the present time, typhoid vaccine, chloral hydrate, sodium cyanide and histamine, and the results obtained may be summarized as follows:

Typhoid Vaccine.—Marine and his coworkers (4) have shown that 75 per cent of rats are killed by 0.25 cc. of a standard typhoid vaccine within 8 days after suprarenalectomy. In a recent paper we showed (6) that suprarenalectomized rats surviving in good condition are killed by typhoid vaccine in doses of 0.4–1 cc. at the height of susceptibility (5–7 days). On repeating these experiments, it was noted

TABLE I.

Effect of Intraperitoneal Injections of Different Batches of Typhoid Vaccine on Suprarenalectomized Rats.

No. of rats	Preparation and Lot No.	Sex	Weight	Interval between operation and injection	Amount of vaccine	Duration life after injection	No. rats killed
			gm.	*days*	*cc.*	*hrs.*	
	N. Y. Board of Health						
3	114–413	F.	165	5	1.0	12	2
		"	172	5	1.0	18	
		"	170	5	1.0	Survived.	
9		"	152	6	1.0	4½	6
		"	150	6	1.0	6	
		"	130	6	1.0	6	
		"	164	6	1.0	5	
		"	144	6	1.0	18	
		M.	192	6	1.0	Survived.	
		"	150	6	1.0	"	
		"	167	6	1.0	"	
		"	190	6	1.0	18	
4		"	180	6	1.5	10	4
		"	184	6	1.5	10½	
		"	178	6	1.5	18	
		F.	160	6	1.5	6	
2		"	132	8	1.0	8	2
		"	152	8	1.0	10	
2		M.	190	8	1.5	7	2
		"	175	8	1.5	18	
9	109–229	F.	135	8	1.0	Survived.	1
		M.	170	8	1.0	8	
		F.	154	8	1.0	Survived.	
		"	120	8	1.0	"	
		"	114	8	1.0	"	
		"	110	8	1.0	"	
		"	124	8	1.0	"	
		M.	179	8	1.0	"	
		"	190	8	1.0	"	
3	115–414	F.	153	7	1.0	"	None.
		"	165	7	1.0	"	
		M.	180	7	1.0	"	

TABLE I—*Concluded.*

No. of rats	Preparation and Lot No.	Sex	Weight	Interval between operation and injection	Amount of vaccine	Duration life after injection	No. rats killed
			gm.	*days*	*cc.*	*hrs.*	
6	115–414	F.	137	7	1.5	5	6
		"	172	7	1.5	18	
		"	167	7	1.5	4	
		M.	189	7	1.5	6	
		"	180	7	1.5	7½	
		F.	145	7	1.5	2	
3*	114–415	F.	157	7	0.2	8½	3
		"	185	7	0.2	18	
		"	192	7	0.2	12–18	
2	U. S. Army triple	F.	195	6	1.5	Survived.	None.
		"	180	6	1.5	"	

* See foot-note 1.

TABLE II.

The Effect of Chloral Hydrate on Normal Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Results
		gm.	*mg.*	*mg.*	
3	F.	150	400	60	Recovered.
	"	146	400	60	"
	"	176	400	70	"
2	"	155	600	93	"
	"	150	600	90	"
3	"	155	700	113	Dead 2 hrs. 10 min.
	"	150	700	105	Recovered.
	"	168	700	117	"
3	"	157	800	125	Dead few min.
	"	155	800	125	" 30 "
	"	153	800	125	" 30 "

that different lots, even of so called standard typhoid vaccine, showed wide variations in toxicity.[1]

Chloral Hydrate.—Eleven normal rats were injected subcutaneously with chloral hydrate. Of three rats injected with 800 mg. per kilo, all were killed. Of three rats injected with 700 mg. per kilo, one was killed, while all five rats injected with 400–600 mg. per kilo survived. Three suprarenalectomized rats were tested at the height of susceptibility (6 days after the removal of the glands), but no decrease in resistance could be demonstrated. See Tables II and III.

Sodium Cyanide.—The subcutaneous M.L.D. of sodium cyanide for normal rats was found to be between 9.8 and 11.0 mg. per kilo, approximately the same dose as that found by Voegtlin, Johnson and

TABLE III.

The Effect of Chloral Hydrate on Suprarenalectomized Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Interval between operation and injection	Results
		gm.	*mg.*	*mg.*	*days*	
3	F.	105	400	42	9	Recovered.
	M.	210	400	84	9	"
	"	210	400	84	9	"

Dyer (7). This amount injected subcutaneously killed five out of six normal rats. Amounts less than 9.8 mg. per kilo did not kill any in a series of six normal rats used. Thirteen suprarenalectomized rats were injected subcutaneously with varying amounts of sodium cyanide. Two rats injected 6 days after removal of the glands

[1] Preparation 114, Lot 415 New York Board of Health vaccine killed with a dose of 0.2 cc. This particular vaccine had been used as a non-specific therapeutic measure in chronic arthritis and cancer, and the clinicians had noted much more marked reactions from a given dose of this preparation than had been obtained with the same dose of other preparations. The variations in toxicity which we had found would explain the differences in results reported by several workers and make typhoid vaccine unsatisfactory as a routine test substance for the resistance of suprarenalectomized rats unless the lethal dose of each lot is separately determined.

TABLE IV.

Effect of Sodium Cyanide on Normal Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Results
		gm.	*mg.*	*mg.*	
3	F.	147	5.5	.80	Recovered.
	M.	164	5.5	.90	"
	F.	138	5.5	.76	"
3	"	144	4.4	.64	"
	M.	184	4.4	.80	"
	"	176	4.4	.78	"
3	F.	136	9.8	1.33	Dead 5 hrs.
	M.	144	9.8	1.40	" 2 "
	"	179	9.8	1.76	" 2 "
3	F.	144	11.0	1.58	Recovered.
	M.	184	11.0	2.01	Dead 1 hr.
	"	176	11.0	1.93	" 1 "

TABLE V.

The Effect of Sodium Cyanide on Suprarenalectomized Rats.

No. of rats	Sex	Weight	Dose per kg.	Amount injected	Interval between operation and injection	Results
		gm.	*mg.*	*mg.*	*days*	
2	F.	150	5.5	.82	6	Killed 3½ hrs.
	M.	180	5.5	.99	6	" 7½ "
2	"	215	2.75	.59	6	Recovered.
	F.	155	2.75	.43	6	"
6	M.	184	4.8	.9	13	Killed 1 hr.
	F.	129	4.8	.63	13	" 18 hrs.
	"	107	4.8	.52	13	" 18 "
	"	132	5.5	.73	13	Recovered.
	M.	150	5.5	.82	13	Killed 1 hr.
	"	166	5.5	.91	13	" few min.
3	"	164	4.85	.8	30	Recovered.
	"	172	4.65	.84	30	"
	F.	121	3.93	.49	30	"

with 5.5 mg. of NaCN per kilo were killed. Two rats injected at 6 days with 2.75 mg. per kilo were not killed. Five out of six rats injected 13 days after suprarenalectomy were killed with 4.8–

TABLE VI, *a*.

Effect of Intravenous Injections of Histamine Acid Phosphate on Normal Rats.

Rat No.	Weight	Histamine acid phosphate per kg.	Remarks
	gm.	*mg.*	
158–2	195	900	Recovered in 3 hrs. In good condition after 2 wks.
158–1	200	1000	Recovered in 3 hrs. In good condition after 2 wks.
158–4	170	1200	Recovered in good condition in 3 hrs. Lived for 6 wks. Died.
169–3	260	1350	Recovered in 3 hrs. In good condition after 2 wks.
158–3	180	1500	Recovered in good condition after 3 hrs. In good condition after 2 wks.
159–2	165	2000	Dead in 2 hrs. 15 min.

TABLE VI, *b*.

Effect of Intraperitoneal Injections of Histamine Acid Phosphate on Normal Rats.

Rat No.	Weight	Histamine acid phosphate per kg.	Results
	gm.	*mg.*	
94	165	1000	Recovered after few hrs. in good condition.
95	232	1200	" " " " " " "
96	150	1300	" " " " " " "
97	195	1400	" " 10 " " " "
98	185	1500	" " 10 " " " "
99	155	1600	Dead after 18 hrs.
100	170	1700	" " 11 "
101	188	1800	" " 4 "

5.5 mg. per kilo. Three rats injected at 30 days with 4.5 mg. per kilo were not killed. It appears therefore that suprarenalectomized rats at the height of susceptibility are about twice as susceptible

TABLE VII.

Effect of Intravenous Injections of Histamine Acid Phosphate on Suprarenalectomized Rats.

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate per kg.	Histamine acid phosphate dose; amount injected	Duration of life after injection	No. of rats killed
		gm.	*days*	*mg.*	*mg.*	*hrs.*	
5	F.	165	20	298.1	49.2	10	5
	M.	200	20	406.0	81.2	½	
	"	220	20	491.7	108.2	¾	
	"	220	20	245.9	54.1	9½	
	"	210	20	351.4	73.8	8	
5	"	220	7	346.3	76.2	5	5
	F.	170	7	520.6	88.5	2¼	
	M.	220	7	171.2	37.9	Few min.	
	"	175	7	140.6	24.6	6¼	
	"	225	7	141.7	31.9	3	
7	F.	165	6	123.0	20.3	6	7
	"	165	6	123.0	20.3	4½	
	M.	200	6	246.0	49.2	2½	
	"	200	6	246.0	49.2	6	
	"	210	6	199.0	41.8	18	
	"	240	6	205.0	49.2	3	
	"	230	6	164.7	37.9	5¼	
3	F.	160	21	187.5	30.0	3	3
	"	165	21	181.8	30.0	12	
	"	175	21	171.4	30.0	18	
4	M.	225	6	80.0	18.0	3	3
	"	243	6	80.0	19.4	3	
	F.	180	6	80.0	14.4	1½	
	"	160	6	80.0	12.8	Survived.	
2	M.	250	6	60.0	15.0	"	0
	"	200	6	60.0	12.0	"	
1	"	240	6	40.0	9.6	"	0
4	"	285	6	20.0	5.7	"	0
	"	185	6	20.0	3.7	"	
	"	230	5	20.0	4.6	"	
	F.	175	5	20.0	3.5	"	
2*	M.	220	7	172.2	37.9	"	0
	F.	195	21	153.8	30.0	"	

* Controls—one adrenal intact.

TABLE VIII.

Influence of the Time Interval after Suprarenalectomy on the Effect of Intraperitoneal Injections of Histamine Acid Phosphate.

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate; amount injected*	Duration of life after injection	No. of rats killed
		gm.	*days*	*mg.*	*hrs.*	
6	M.	200	2	20.0	Survived.	1
	F.	165	2	16.5	2	
	"	200	2	20.0	Survived.	
	"	190	2	19.0	"	
	"	195	2	19.5	"	
	"	170	2	17.0	"	
5	"	220	3	22.0	"	2
	"	220	3	22.0	12	
	M.	250	3	25.0	Survived.	
	"	240	3	24.0	48	
	"	260	3	26.0	Survived.	
7	"	230	4	23.0	"	2
	F.	190	4	19.0	"	
	"	180	4	18.0	13	
	"	200	4	20.0	Survived.	
	"	165	4	16.5	"	
	"	190	4	19.0	"	
	M.	220	4	22.0	2	
12	"	190	5	19.0	2	12
	"	220	5	22.0	3	
	"	210	5	21.0	18	
	"	179	5	17.9	1	
	"	194	5	19.4	1	
	"	181	5	18.1	1	
	"	181	5	18.1	$2\frac{1}{2}$	
	"	201	5	20.1	$\frac{1}{2}$	
	"	193	5	19.3	3	
	F.	168	5	16.8	3	
	"	132	5	13.2	4	
	"	162	5	16.2	9	

* 100 mg. per kilo of body weight.

TABLE VIII—*Concluded.*

No. of rats	Sex	Weight	Interval between operation and injection	Histamine acid phosphate; amount injected*	Duration of life after injection	No. of rats killed
		gm.	*days*	*mg.*	*hrs.*	
10	F.	180	6	18.0	2	8
	"	200	6	20.0	18	
	M.	240	6	24.0	4	
	"	218	6	21.8	Survived.	
	"	240	6	24.0	1	
	F.	185	6	18.5	2	
	M.	210	6	21.0	Survived.	
	F.	159	6	15.9	1	
	"	150	6	15.0	2	
	M.	216	6	21.6	1½	
6	F.	200	7	20.0	Survived.	3
	"	210	7	21.0	"	
	"	160	7	16.0	2	
	"	225	7	22.5	1	
	"	190	7	19.0	Few min.	
	"	190	7	19.0	Survived.	
3	"	250	8	25.0	2	1
	"	180	8	18.0	Survived.	
	"	240	8	24.0	"	

to NaCN as are normal rats. This difference in the lethal dose of NaCN for suprarenalectomized and normal rats is, however, so small that this drug cannot be advantageously used as a test for decreased resistance. See Tables IV and V.

Histamine.—the increased susceptibility of suprarenalectomized animals to histamine has been shown by numerous investigators (8–12).

The intravenous M.L.D. of histamine acid phosphate for normal rats has been found to be approximately 900 mg. per kilo by Voegtlin (13) and 500–700 mg. per kilo by Crivellari (11).

In our experience (Tables VI,*a* and VI,*b*) the M.L.D. for normal rats is approximately 1600 mg. per kilo. As the preparation of histamine used in our experiments was the same as that employed in

the experiments of Voegtlin (13) and Crivellari (11) it is possible that the difference in the lethal dose is due to a difference in the age or in the susceptibility of the albino rats that we and they have used. Our rats were approximately 5 months of age and were reared in the laboratory from Wistar stock.

The increased susceptibility of suprarenalectomized rats to the intravenous injection of histamine is shown in Table VII. As the intravenous M.L.D. for these animals is approximately 80–100 mg. of histamine acid phosphate per kilo the suprarenalectomized rats are about 20 times more susceptible to histamine than the normal rats of the same age and strain. Banting and Gairns (10) found that suprarenalectomized dogs are about thirty times more susceptible to histamine than are normal dogs.

As the intravenous injection of histamine acid phosphate in doses as high as 2000 mg. per kilo involves the injection of comparatively large amounts of phosphoric acid, it seemed advisable to exclude by control experiments a possible effect of phosphoric acid. We therefore injected two normal rats with the amount of phosphoric acid per kilo contained in 2000 mg. of histamine acid phosphate per kilo. Instead of the phosphoric acid solution, an equivalent amount of $NaH_2PO_3 \cdot H_2O$ was used. The rats were sick for about 3 hours but recovered completely. Smaller amounts of $NaH_2PO_3 \cdot H_2O$ (1/10th of the dose used for the normal rats) had no effect on two suprarenalectomized rats.

The results of intraperitoneal injections of histamine acid phosphate in 49 suprarenalectomized rats are given in Table VIII. Of 18 suprarenalectomized rats injected with 100 mg. of histamine acid phosphate per kilo between the 1st and 4th days after the removal of both glands, 5 were killed, whereas 24 out of 31 suprarenalectomized rats injected at 5, 6, 7 and 8 days after the operation were killed.

A comparison of Tables VII and VIII shows that the difference in the M.L.D. of histamine acid phosphate for suprarenalectomized rats when given intravenously and intraperitoneally is negligible. Since the technique of intravenous injection in the rat is quite difficult, these results show that the intravenous method may be abandoned for this purpose.

We should like to call attention again to the latent period after

suprarenalectomy. As pointed out previously (6), the great drop in resistance does not occur until about the 5th day after the operation. This is illustrated in Table VIII where of 18 rats injected between the 2nd and 4th days, only 5 were killed, whereas of 31 rats injected with the same amounts between the 5th and 8th days, 24 were killed.

SUMMARY.

Of all the drugs thus far used in testing the resistance of suprarenalectomized rats, histamine has been found to be the most satisfactory. It is a readily obtainable and comparatively stable drug, producing characteristic symptoms. Its M.L.D. for a given strain of rats can be established within narrow limits, and the difference between the M.L.D. for normal and suprarenalectomized rats is comparatively great (1:20).

We wish to thank Dr. David Marine for his helpful advice and criticisms throughout the course of this work.

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LOCAL SPECIFIC THERAPY OF EXPERIMENTAL PNEUMOCOCCAL MENINGITIS.

IV. Type II Pneumococcal Meningitis in Dogs.

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Plates 24 to 28.

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This paper is the fourth of a series of studies on experimental pneumococcal meningitis. The three preceding (1–3) are concerned with various aspects, both pathologic and therapeutic, of experimental Type I pneumococcal meningitis in rabbits and dogs, together with a discussion of certain complicating factors in the disease. The pathologic studies are too extensive to summarize at this instance. From the point of view of therapy, however, it may be said that in one of these papers (2) a method was described whereby in suitable, uncomplicated cases, a number of permanent cures resulted. This method consisted in a saline lavage followed by a generalized flooding of the meninges with mixtures of immune serum and ethylhydrocupreine hydrochloride. The most important factor in obtaining successful recovery from the disease was our ability to bring repeatedly all infected regions of brain and cord into contact with the serum-drug mixture. This possibility, in turn, was favored by the nature of the early pathologic reaction on the part of the animal. With the pneumococcus strains employed, the animals reacted with only a moderate early leucocytosis; the fibrin was *relatively* scanty and there was a strong tendency of the spinal fluids toward the assumption of the picture which the French describe as "dissociation cytobactériologique du liquide céphalorachidien," *i.e.*, a fluid with many organisms yet a relatively mild fibrinocellular reaction. In other words, therapeutic possibilities were excellent. The exudate was not so fibrinous as to preclude extensive lavage and good serum-drug contact with the

pneumococcus. Although the organisms at first were present in very large numbers, after the lavage they were greatly diminished and following serum injection the degree of phagocytosis was very marked. As the disease progressed, *with or without treatment*, in certain of the animals a degree of partial immunity was apparently established and its expression was evidenced by a massive leucocytosis; this leucocytosis, however, was not very effective, since no matter how many leucocytes were present in the spinal fluids, the phagocytosis, in the absence of immune serum, was slight. This very leucocytosis, instead of favoring recovery under treatment, actually prevented effective treatment, since under these conditions lavage became impossible and injected serum failed to distribute itself properly. A successful treatment was one in which, by lavage, very early reduction in the number of organisms occurred, in which serum-optochin injections brought about active phagocytosis, and which, by repetition, prevented subsequent development of organisms to an extent sufficient to excite these massive purulent reactions.

It is a well established fact that the potency of most Type II antipneumococcus serum is low. Nevertheless, since in meningeal disease the therapeutic agent is introduced intrathecally into direct contact with the infecting agent, it was deemed reasonable to attempt the therapy of Type II meningitis in a manner similar to that employed in the treatment of Type I. It may be stated at the outset that we were unable to cure any of the animals. The reason for this failure will be readily apparent from the following description and discussion of the work.

Course and Pathology of Disease.

Four strains of Type II pneumococcus were used in the production of the disease. In all instances a Petroff-Hausser counting chamber served to determine dosage. As a rule 6 hour dextrose broth cultures were employed and the amount injected was usually from 0.5 to 1.0 cc. of a 1:100 culture dilution. No attempts were made to alter artificially by serial animal passages the "virulence" of the strains; every strain was transferred on receipt to blood broth and kept as stock, transplants being made to dextrose broth and incubated about 6 hours before use. Growth was always very heavy. One of the strains (II, 62) was of low activity in the dog, as appears from the following protocols.

Dog 1.—Female hound; weight 4 kilos. October 13, 1927, 4.35 p.m., cistern

puncture under ether; clear fluid. Injected 1.7 million Type II pneumococci, Strain II, 62. Normal ether recovery.

18 hours: Apparently well.

23 hours: Ether; cistern puncture; slightly cloudy fluid; rare cells and no cocci in smears. Culture negative. Subsequent uneventful recovery.

Dog 2.—Hound; weight 5 kilos. October 17, 1927, 4.00 p.m., cistern puncture under ether; clear fluid. Injected 10 million Type II pneumococci, Strain II, 62.

19 hours: Slightly sluggish; slight ataxia. Ether, lumbar and cistern taps; drained 3 cc. very cloudy fluid under tension from cistern; smear showed numerous polymorphonuclears per oil immersion field, but no cocci were seen. Upon planting a large amount of the fluid on a blood agar plate only fifteen colonies resulted. A spasmodic muscle contraction resulted in cisternal hemorrhage with death. Autopsy showed, aside from the area of hemorrhage, a grossly normal brain; no cocci were seen in any of the smears. Cultures from all regions were negative, except that a plate planted with the entire content of one lateral ventricle grew eight colonies.

Other animals infected with this strain made it appear that the strain activity was not such as to render its use desirable in our work. Some strains of Type II are apparently of even much lower activity, as demonstrated by the work of Idzumi (4). Idzumi infected his dogs subthecally with very large amounts of a Type II pneumococcus (8 cc. of a 24 hour broth culture lethal for mice in 50 to 60 hours in a 10^{-5} dilution). This dosage killed the dog in 20 hours. Only one experiment is reported and the pathological findings after this very large dose do not especially differ from our own, when using much smaller infecting quantities.

Two of our strains designated as D 39 and II 740 produced pictures identical in most respects. If anything, II 740 was somewhat more active. Bacteremia was rarely a problem; a few colonies from the postmortem heart's blood were a frequent finding. When significant bacteremia did occur, it was apparently the result of some individual peculiarity; it was—of course within reasonable limits—independent of weight of animal and infecting dosage, as the following two protocols demonstrate.

Dog 3.—Hound; weight 3.5 kilos. May 19, 1927, 10.45 a.m., cistern puncture under ether. Injected 18.0 million Type II pneumococci, Strain D 39. Anesthesia continued with intravenous sodium diethylbarbiturate. Samples of

blood were withdrawn at intervals from the leg vein and planted in 1 cc. quantities on blood agar plates with the following results:

1 hour	no growth.
3½ hours	no growth.
5½ hours	5 colonies per cc.

22 hours after infection the dog was found dead. Autopsy revealed generalized purulent meningitis; the entire brain was covered with yellowish, adherent, exudate. Abundant cloudy fluid in the cord meninges; cortex and cord showed petechial hemorrhages. Organisms were present everywhere, but never as numerous as one finds in the animal dying of Type I infection; especially in the cisternal pus there was very marked phagocytosis and one could observe many foci of pseudoagglutination. (We have coined the term "pseudoagglutination" to describe the appearance of exudates containing clusters of pneumococci formed by growth of groups of cocci recently liberated by the degeneration of leucocytes which had previously phagocyted them.) The heart's blood gave slight growth. The lungs showed congestion and edema.

Compare this with the following protocol of an experiment performed with the same culture, the same day, on a dog of the same weight and litter. The infecting dose was only half that used in the preceding case, yet there was early generalization of the disease.

Dog 4.—Hound; weight 3.5 kilos. May 19, 1927, 11.00 a.m., cisternal injection under ether of 9.0 million Type II pneumococci, Strain D 39. Anesthesia was continued with intravenous sodium diethylbarbiturate. Interval bleedings gave the following results:

1 hour	no growth.
3½ hours	no growth.
5½ hours	1050 colonies per cc.

At 22 hours the animal was found dead. Autopsy showed a dry, hyperemic brain; the exudate was scanty; cells and cocci were rare. In addition there were acute seropurulent pleuritis and peritonitis; pneumococci were demonstrated in the fluids both by direct examinations and by cultures. The heart's blood culture gave massive, confluent growth.

Only twice during the study of Type II meningitis have we felt that there was sufficient generalization of infection to modify the prognosis of the disease. These infecting doses given in the two preceding experiments were somewhat larger than those subsequently employed. In brief, infecting amounts of 18, 15, and 12 million organisms of the D 39 strain given intracisternally killed in less than 1

day. Smaller numbers have given more inconstant results. Doses of 7 million organisms in three experiments permitted survival up to 40, 42, and 66 hours respectively; however, all of these survivals received one or more treatments which may possibly have delayed death. Intracisternal doses of 3 to 3½ million pneumococci usually allowed survival beyond the 1st day of disease, but even with this low dosage some animals have succumbed within 18 hours. The lower the infecting dose, the more apparent are the individual differences as regards the period of survival. Obviously a dosage which is quite apt to kill in less than 1 day is not a suitable one for the production of a type of disease which may be studied from the point of view of therapy.

Of course we can offer no explanation for individual differences in the survival period of the animals. There are, however, certain facts which correlate these differences with the pathological findings in the different animals. When the same amounts of the same culture are used for infection, it is impossible to see how the pathology can be explained by any difference in the infecting organism. The only variant is the response of the animal and the varying intensity of this reactional process is very important; this is naturally true in any infectious disease, but perhaps it is most apparent in central nervous system infections of the variety under study, because the nature and relations of the brain and cord make them unsuitable to withstand any severe, acute, reactional pathology.

If one infects a dog intracisternally with Type I pneumococcus and subsequently examines the spinal fluid, one notes, after a brief period during which time the organisms diminish somewhat in numbers, due in part to a slight initial phagocytosis, a progressive increase in organisms. Phagocytosis becomes negligible and the picture is one of cytobacteriologic dissociation. In Type II infections, from the start there is rather marked phagocytosis of cocci, even approaching in degree that seen after serum therapy of Type I. Coupled with this primary resistance to Type II there is a very heavy exudation of fibrin and polymorphonuclear leucocytes, and a tendency toward localization of the disease; this attempt at localization is never, in our experience, completely successful, but its effect may be disastrous to the animal, as the following protocol demonstrates.

Dog 5.—Male collie pup; weight 4¼ kilos. September 27, 1927, 4.25 p.m., morphine; ether; cistern puncture; clear fluid. Injected 3.5 million Type II, Strain D 39. Normal ether recovery.

18 hours: Was just expiring when cage was visited. Autopsy: cord meninges edematous, injected, hemorrhagic, filled with fibrinopurulent exudate. Smears show numerous pus cells and a moderate number of diplococci, with evidence of considerable phagocytosis, coccal disintegration, with some small, atypical, and Gram-negative forms. Grossly about the medulla, cistern, and pontile base, the exudate is very thick and so fibrinous that it must be stripped off with forceps. The medullary smear resembles that from the cord. Convexity and base show much hemorrhagic injection (Fig. 1). Along the veins of the convexity is a moderate amount of cloudy fluid, containing fairly numerous cocci. The lateral ventricles contain abundant opalescent fluid, with rare cells but numerous cocci. There is slight bacteremia.

Fig. 2 gives some indication of the fibrinous character of the exudate at the site of injection. The fibrin distribution is that characteristic of those cases which die early after cistern infection; there is a very heavy layer closely surrounding the cord or medulla, and this layer continues out in similar fashion about the nearby vessels; this dense fibrin is comparatively devoid of cells, but further out, where the fibrin meshes are less dense, polymorphonuclear leucocytes are abundant. Curiously enough, by Gram-Weigert staining few pneumococci are found in the dense fibrin layers, but in the looser, fibrinocellular portion they are abundant. The vessels may show considerable damage and frequently, from serum exudation into their walls, they stain in a hyaline manner; the vessel walls are extensively infiltrated by polymorphonuclear leucocytes. Occasionally with these marked reactions it is possible to detect deep changes in the nerve cells of the cord. These changes consist in necrosis with polymorphonuclear reaction and invasion of the affected nerve cells (Fig. 11). The smaller vessels of the gray matter show some polymorphonuclear perivascular reaction, and there is slight generalized, or patchy infiltration (Fig. 12) of the interstitial nerve tissue either in the cornua or fasciculi. This is not the same phenomenon which one sees in a spreading myelitis of central canal origin, for in the former it has not been possible to demonstrate organisms in the region or in the finer vessel sheaths, whereas in the latter—the central myelitis—organisms are very abundant and the pathological picture is quite different. The pathology suggests the action of a diffusible toxic substance; were it not for the marked

meningeal reaction, one might easily confuse the picture with poliomyelitis. In the protocol of Dog 5 it is seen that the organisms were rather numerous; the infection at death was severe; changes in deep nerve cells may have hastened death, but in other animals death has occurred without a marked proliferation of organisms, without massive ventricular growth, without detected deep nerve cell pathology, and in the absence of significant bacteremia. Nothing, therefore, is left to account for death, except the presence of the massive exudate in the cisternal region adjacent to vital centers, and experience forces us to attribute to this reaction very unfavorable significance. We noted in Type I pneumococcal meningitis the severity of symptoms for a few hours after extensive serum treatment and were inclined to attribute it to the marked increase in leucocytosis—a transient phenomenon. Had a general anesthetic not been necessary, we would have drained our animals several times at the height of the leucocytosis to relieve the intensity of the reaction and it is suggested that in the treatment of human disease this procedure would be desirable.

The effect of a massive cisternal exudate is apparent in another way. The space is small and a block is readily attained. As a rule, the lateral ventricles are infected early in the course of the disease. A primary cisternal injection in the amount used in our experiments does not pass into the lateral ventricles; as judged by dye injections in equivalent quantities, it distributes itself to the cervical cord, fourth ventricle and its meninges, and to the base and adjacent lower lateral portions of the cerebrum; the cerebellum may be stained, but the tentorium prevents forward spread to the convexity. Apparently infection follows the same course. A marked early, cisternal block may occur, with organisms present in the cisternal exudate but without cocci in the lateral ventricles at the time of death. In the absence of early block the ventricles may become infected very soon in the course of the disease, and in this case the pneumococci multiply to an extraordinary extent in the lateral ventricles behind the subsequently developing block, and fairly well established hydrocephalus may develop within 2 days after primary infection. An interventricular block may result in quite different pictures in the two lateral ventricles. The following two protocols show the probable effect of cisternal block on the distribution of the infecting agent.

Dog 6.—Hound; weight 8.4 kilos. October 24, 1927, 4.35 p.m., ether; cistern puncture; clear fluid. Injected 5 million Type II pneumococci, Strain D 39.

20 hours: Dead. Autopsy reveals the usual fibrinohemorrhagic exudate blocking the fourth ventricle and extending over medulla and cervical cord; small amount of purulent exudate at the base; lower cord and convexity practically clear in gross; the lateral ventricles contain abundant opalescent fluid with pus cells but no cocci; cervical cord and cisternal smears show abundant pus cells and a few free and phagocyted cocci. A "reactional" death.

Dog 7.—Fox terrier; weight 5 kilos. October 19, 1927, 4.15 p.m., ether; cistern puncture; clear fluid. Injected 2¼ million D 39 Type II pneumococci.

18 hours: Dead. Autopsy reveals much fibrin and some clot in fourth ventricle. Cord and base show moderate purulent exudate. Softening in posterior vermis. Cortex dry and injected. Abundant cloudy fluid in lateral ventricles. All smears show numerous pus cells, but the cocci are rare and nearly all phagocyted, except in the lateral ventricles, where they occur free in enormous numbers (Fig. 5). Cultures from lateral ventricles yield confluent growth; from other regions a few colonies only. Slight bacteremia.

Block is of importance in influencing the distribution of organisms, especially when it occurs in the cisternal region; its influence in other situations has not been determined, but is probably less. Its significance at the cisterna is most apparent when infection passes the early block and invades the lateral ventricles, for it has been abundantly demonstrated that in the ventricles with a favorable fluid medium the organisms grow to an enormous extent, whereas in less favorable regions, base, cord, and convexity, there is some tendency to spontaneous disappearance partially, at least, through phagocytosis. In the lateral ventricles the rapid growth of pneumococci seems to repel phagocytosis and it is not seen save to a minimal degree in the absence of immune serum. Figs. 6 and 7 are from the autopsy of a dog infected in the lateral ventricle. The animal died in 18 hours; there was no blocking exudate in the cistern; the foramina of Munro may have been occluded and this is probable since the picture from the two lateral ventricles was not the same. Some organisms, however, have passed through into the cistern and meninges, and the smears, as is seen, are quite different; in one there is abundant growth and little or no phagocytosis, whereas in the other—from the cistern—phagocytosis is marked and cocci are few. Therefore, it is apparent that even without block the rapid proliferation of organisms in the lateral ventricles fades out as the cisternal and cord regions are reached. What block

does is to delay lateral ventricle infection, if the block occurs early, and to place a barrier between a site of prolific growth and a region of much less active multiplication, if it occurs only after the ventricle is infected. Some of these cells in the cistern smear (Fig. 6) are endothelial leucocytes. It is easily shown that the endothelial leucocyte readily phagocytes Type II pneumococci in the dog. This was particularly apparent in the case of a dog infected in the basal region; the animal developed a pneumococcus brain abscess along the needle track (Fig. 9). Many endothelial leucocytes were attracted to the softening areas at the periphery of the abscess, and all of these are loaded with pneumococci, many of which are fragmented. Toward the center of the abscess, where there are only polymorphonuclear leucocytes, pneumococci are abundant and there is little or no phagocytosis. When softening develops, as it may in foci in a meningeal exudate, endothelial leucocytes may appear at the periphery of softened areas and actively phagocyte pneumococci; in these rare foci of softening in the meningeal exudate, however, the pneumococci tend to disappear; they are probably injured by the mass liberation of ferments from the degenerated leucocytes.

We have discussed the influence of the massive fibrinopurulent exudate in the cisterna on life and on the distribution of infection. It is interesting to note the effect of this type of reaction in other regions. The very large fibrin content of the exudate tends to be maintained everywhere, except in the lateral ventricles, where either the process results in a purulent reaction, or a type resembling the "dissociation cytobactériologique" common in Type I. Next to the cistern the penetrating vessels suffer most markedly from the character of the exudate. This is apparent, though by no means uniform, in several regions,—cord, convexity, and thalamus. We have followed the process by ordinary methods and by Gram-Weigert staining, and are inclined from our observations to interpret it as follows: Organisms invade the penetrating vessels and their perivascular sheaths from the meninges. The reaction consists in marked polymorphonuclear leucocytic invasion of the vessel walls and an exudation of serum, fibrin, and leucocytes into the sheaths and surrounding parenchymatous tissue. The reaction takes place in the vessel walls and perivascular sheaths in advance of detectable invasion by the pneumococci, and it

is possible to observe, by Gram-Weigert staining, pneumococci to a certain level, marked reaction somewhat deeper still, and not infrequently thrombosis of the smaller branches. Fig. 13 illustrates the process in the deeper cord vessels; Figs. 14 and 15 in the thalamus; in the latter a smaller branch is thrombosed and resultant changes in the surrounding parenchyma have attracted leucocytes into the brain tissue. This region as yet is apparently uninfected, but when organisms finally do penetrate into the focus, they proliferate rapidly in the injured brain tissue and readily convert an area damaged by thrombosis into a pneumococcus abscess. In the cord region central myelitis is common. Vessel sheaths are early involved by the infectious process originating from the central canal (3) and central softening occurs; a similar type of lesion spreads in along vessels, penetrating from the meninges, and the combination destroys large portions of spinal cord. In the central myelitis the first organisms reach the cord tissue through the ependyma (Fig. 16). Once in the parenchyma they invade extensively between the swollen, damaged, myelinated fibers (Fig. 17).

Often considerable hemorrhage is present. When this occurs, it is usually in the cisternal region and for a while, although our initial cisternal taps at the time of infection gave clear fluid, we were inclined to attribute it to trauma. Later, however, it was noted in other regions, notably choroid plexus and convexity. Fig. 10 gives some notion of the extreme vasodilatation one may encounter in the choroid plexus of the lateral ventricles.

All of the preceding description refers to the sequences of primary cisternal infection. We were forced to abandon this method of inoculation because of the severity of the local reactions. Other methods of infection were next employed. One animal was infected in the lateral ventricle in the hopes that the infection would be distributed more gradually *via* the usual cerebrospinal fluid pathways, thus avoiding early severe, local reaction in the cisternal region; the method was worse than useless, for not only did it fail to prevent rapid spread to the cistern, but in addition it placed the pneumococci immediately in that region in which we have determined their growth to be most rapid and luxuriant. Fig. 7 illustrates the condition 18 hours later in the lateral ventricle. Infection directly through the brain to the

base was attempted in several instances. It was a first impression that this produced a more favorable type of disease, but in reviewing the records this opinion scarcely seems justified. Organisms introduced into the basal meninges do not tend to proliferate markedly at that site, or to spread in large numbers to the convexity. Instead they penetrate to the cisterna and thence to lateral ventricle and cord. In making this statement, we cannot be certain that initial lateral ventricle infection has not occurred along the needle track. Our assertion is merely based on numbers of organisms in different regions. In basal infection brain abscess may occur along the needle track. Primary infection of the convexity produced a severe clinical picture with marked depression, opisthotonos, spasticity of all four limbs, vomiting, and respiratory difficulty within 20 hours after infection. Death occurred during the 2nd night; the meningitis was already generalized, fibrinopurulent in character, but, although death occurred early, organisms were exceedingly rare and largely phagocyted. Death, we believe, was "reactional." Attempts at infecting the animals in the lumbar meninges are best taken up with our discussion of therapy. Infection by the lumbar route spreads more slowly than it does by the other routes employed.

Therapeutic Experiments.

The method employed in treating the animals which survived long enough to make a therapeutic effort feasible was the same as that already described for the treatment of Type I meningitis (2). Studies of the distribution of the process made us, however, abandon direct serum-drug treatment of the convexity since that region, due to localizing efforts on the part of the animal, was only rarely involved. As we have stated, the lateral ventricles early become the chief reservoir of pneumococci; lateral ventricle punctures were therefore used rather extensively both for drainage and serum-optochin injections. The serum employed was kindly furnished by Dr. Wadsworth, of the New York State Department of Health. The following series of protocols are offered as examples of therapeutic experiments. It will be readily noted that the possible degree of success apparent in some instances is negatived by the complete failure in others. Indeed, it may be stated

that treatment appeared without value, even when it was carried out so early in the course of the disease that diagnostic punctures at the time the treatment was initiated not only failed to reveal pneumococci in the fluid smears, but likewise in cultures. If success had been attained in such instances, it would be of little significance, since no human case could possibly be diagnosed and typed at so early a period and the disease is one in which a few hours of progress make a profound difference in the picture.

Dog 8.—Hound; weight 4½ kilos. November 2, 1927, 4.30 p.m., morphine; ether anesthesia; skull trephine; puncture through to left base; slight hemorrhage. Injected 3½ million, Type II, D 39 strain. Normal recovery.

18 hours: Sluggish; chills; no meningeal signs.

24 hours: Still stuporous from morphine. Has slept all day. On disturbing, stands up, walks, and wags tail.

42 hours: Apparently well. No treatment.

66 hours: Very ill; irritable; lies prostrate on side; all extremities spastic with exaggerated reflexes. Lumbar and cistern puncture under ether. No fluid obtained *via* lumbar needle, but withdrew 1.5 cc. very thick purulent exudate of gelatinous consistency from cisterna. A smear showed large numbers of polymorphonuclear leucocytes and pneumococci (Fig. 4). On account of the thickness of the exudate, no saline lavage was possible. The usual serum-optochin mixture was prepared (10 cc. serum plus 0.5 cc. 1 per cent ethylhydrocupreine hydrochloride) and 4 cc. were injected, half in the cisterna and half in the lumbar region. After a few minutes rest both lateral ventricles were entered through trephine openings and the contents aspirated. Apparently the immune serum injected into the cisternal region had already reached the lateral ventricles, since the abundant pneumococci were all agglutinated (Fig. 8); cells were very scanty; 0.6 cc. serum-optochin injected in each lateral ventricle and 0.6 cc. over each convexity. Recovery rate difficult to detect on account of initial prostrate condition. Placed in constant body temperature cage and removed to animal house after some hours.

90 hours: Dead. Autopsy shows heavy fibrinopurulent and hemorrhagic exudate over cord and about cistern; exudate scanty at base, where initial infection was introduced. Small amount of pus in lateral ventricles. Smears from all regions show very numerous pus cells; cocci are diminished in numbers (Fig. 3) and where numerous show agglutination. There is little evidence of stimulated phagocytosis. Small abscess of medullary floor (traumatic?). Beginning hydrocephalus.

This animal probably had his pneumococci reduced by the treatment, but it is difficult to ascertain truly if this was the case. Other experiments at least throw considerable doubt into the matter.

Dog 9.—Hound; weight 4½ kilos. September 29, 1927, 4.20 p.m., morphine; ether; cisternal tap; clear fluid. Injected 1¾ million Type II D 39. Normal recovery.

18 hours: Temperature 102.7°. Stupid and refuses to stand up. Raises head and is irritable when touched.

21½ hours: Ether; cistern and lumbar punctures; fluids cloudy; contain 25–30 polymorphonuclears per oil immersion field; no cocci seen in direct smear. Cultures: growth but not confluent. Lavage of 5 cc. NaCl, lumbar to cistern, with good return. Distributed 11 cc. serum-optochin mixture as follows: 8 cc. cord to cistern, 1.5 cc. frontals *via* trephines. Satisfactory recovery.

44 hours: Temperature 102°. Has lost weight; condition otherwise unchanged. ether; lumbar and cistern punctures; fluid clearer; cells rare; many mononuclears; no cocci seen. Injected 5 cc. serum-optochin *via* cord and cistern. Cultures negative.

Subsequent uneventful recovery.

The above protocol shows the influence of treatment but only in a prophylactic way. At the time therapy began the case was not capable of being diagnosed. As we have intimated, even as a prophylactic, the serum may fail to do anything save to delay the inevitable, as the following example clearly demonstrates.

Dog 10.—Weight 5½ kilos. December 5, 1927, 4.30 p.m., ether; lumbar puncture; clear fluid; injected 2 million Type II pneumococci, Strain D 39. Good recovery.

19 hours: Weakness in lumbar region; slight tremors in hind limbs; otherwise normal.

23 hours: Unchanged. Ether; lumbar puncture; cloudy fluid; cells 15–20 per oil immersion field; no cocci seen. Cisternal fluid clearer; few cells and no cocci. *Neither fluid gave a positive culture.* Lavage with 10 cc. NaCl lumbar to cistern; return unsatisfactory, but no pressure symptoms. Injected as usual 10 cc. serum. (No drug was used, since without much exudate and with poor fluid return there is great danger.) Both lateral ventricles were entered and, after withdrawal of a practically negative fluid already mixed with serum, 1 cc. of additional serum was introduced. Lateral ventricle culture gave no growth.

42 hours: Unchanged. No treatment.

66 hours: No treatment. Appears well.

7th day: Has suddenly become very ill. Necessary to chloroform. Immediate autopsy. Massive, generalized meningitis; complete block at the cisterna; hemorrhagic flecks over convexity. Every smear shows very numerous pus cells. Cocci are very abundant in the lateral ventricles, numerous in the cisterna, few at the base, and very few over the convexity and in the cord meninges. The heart's blood yields rare colonies.

Summary: Cord infection; extensive early treatment when organisms were so few that they were not detected by culture, yet the fatal outcome of the disease was merely delayed.

The objection may be raised that treatments in cases such as that summarized above were not systematically continued. In the absence of organisms in the initial cultures and smears, it would have been quite illogical to treat. In the following protocol the effect of repeated treatments is shown.

Dog 11.—Hound; weight 6 kilos. November 21, 1927, ether; left frontal trephine; puncture through to base and injection of 2 million Type II pneumococci, Strain D 39. Normal recovery.

18 hours: Ataxic; irritable; able to stand. Morphine; ether; cistern and lumbar punctures; fair drainage; fluids opalescent, containing on smear about 25 polymorphonuclears per oil immersion field; cocci very rare. Injected 3 cc. serum-optochin cord, 2 cc. cistern, 0.6 cc. right lateral ventricle, 1 cc. left lateral ventricle, and 0.5 cc. subdurally over each convexity. Some shock but recovery in 45 minutes. Cultures positive.

42 hours: Clinically unchanged; possibly slightly improved. Cistern and lumbar punctures under ether. Fluids less cloudy; cells diminished but cocci slightly more numerous; they are both free and phagocyted, and occur singly and in rare clusters of 5 or 6. Injected 4 cc. serum-optochin cord, 1 cc. cistern, and, after draining, 1 cc. in each lateral ventricle; smears from the ventricles show occasional free cocci. Cultures yield confluent growth.

66 hours: Unchanged except for loss of weight. Ether; cistern and lumbar punctures; fluid slightly clearer; cells diminished; no cocci seen. Lavage with 5 cc. serum-optochin lumbar to cistern; lumbar needle removed and 1 cc. additional injected into cistern. Left lateral ventricular puncture; fluid practically clear; smear shows rare cells but no cocci. Injected 0.5 cc. serum-optochin. Cultures give slight growth.

90 hours: Animal worse; more irritable. Ether; no cistern fluid obtained; no lumbar fluid. Nevertheless, 3 cc. of serum-optochin was injected. The left lateral ventricle was entered and 0.5 cc. of what appeared in gross as clear serum was withdrawn. 1 cc. serum-optochin injected. Smears of this apparent serum show essentially no cells but numerous clusters of agglutinated pneumococci. Culture yields a confluent growth.

114 hours: Prostrate; convulsive; very hypersensitive. Chloroformed. The usual autopsy findings. Massive fibrinopurulent exudate in and about cistern, at base, and in lateral ventricles. Cord and convexity are relatively clear. Smears show hundreds of leucocytes and pneumococci per oil immersion field. Little phagocytosis except in cord and cisternal regions. Hydrocephalus. Multiple brain abscesses along basal and lateral ventricular needle tracks.

In other words, systematic treatments extended over several days failed to cure a very slight initial infection. It undoubtedly did delay death.

SUMMARY AND CONCLUSIONS.

1. With properly selected strains of Type II pneumococci, fatal meningitis may be produced by intrathecal infection in dogs.

2. The pathology of the disease differs from that produced by Type I pneumococci, at least with the strains employed.

3. Type II meningitis is characterized by early, very marked reactions, a very heavy fibrin exudation, and a tendency toward the rapid establishment of blocks.

4. Extreme vasodilatation and hemorrhage are common.

5. The fibrinopurulent exudate tends to involve the walls and perivascular sheaths of deeply penetrating vessels, thereby causing thrombosis.

6. Thrombosis leads to foci of parenchymatous softening which, on subsequent infection, become brain abscesses.

7. The lateral venticles are a "point of election" for the luxuriant growth of pneumococci. Conversely there is evidence that Type II organisms tend somewhat to disappear from other regions, owing either to spontaneous phagocytosis or to filtration into the blood stream. The lateral ventricles, however, continually renew the supply.

8. Hydrocephalus occurs early—sometimes during the 2nd day of the disease.

9. Central myelitis is common and appears early in the disease.

10. Deep nerve cell lesions with necrosis and neuronophagia are encountered. Their pathology suggests a toxic source.

11. In most cases treatment has been impossible owing to severe early reactions, and only as a prophylactic has optochin-serum therapy any real value. It delays death, but has in no instance cured established disease in the dog.

12. Systemic generalization of Type II meningeal infection in the dog occurs, but it is rarely of sufficient extent to modify prognosis.

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EXPLANATION OF PLATES

PLATE 24.

FIG. 1. Lower aspect of brain. Fibrinopurulent exudate beneath dura, extending over cervical cord and forward toward infundibulum.

FIG. 2. Dog 5. Typical massive, fibrinopurulent exudate. Weigert stain. × 100.

PLATE 25.

Fig. 3. Dog 8. Autopsy smear from cisterna. Gram stain. × 1000.

FIG. 4. Dog 8. Initial smear from cisterna. Gram stain. × 1000.

FIG. 5. Dog 7. Autopsy smear from lateral ventricle. Gram stain. × 1000.

FIG. 6. Dog 12. Autopsy smear from cisterna. Gram stain. × 1000.

PLATE 26.

FIG. 7. Dog 12. Autopsy smear from lateral ventricle. Gram stain. × 1000.

FIG. 8. Dog 8. Agglutination of pneumococci. Gram stain. Lateral ventricular tap. × 1000.

FIG. 9. Needle track abscess. Dog 11.

FIG. 10. Prehemorrhagic dilatation of vessels of choroid plexus of lateral ventricle. Hematoxylin-eosin stain. × 40.

PLATE 27.

FIG. 11. Dog 5. Spinal cord. Necrosis and early neuronophagia of motor nerve cells. Hematoxylin-eosin stain. × 100.

FIG. 12. Dog 5. Spinal cord. Leucocytic infiltration of white matter in region of radix descendens V. Hematoxylin-eosin stain. × 100.

FIG. 13. Dog 13. Spinal cord. Deep vascular lesions; polymorphonuclear invasion of vessel wall and serofibrinous exudation in and about vessel sheath. Hematoxylin-eosin stain. × 100.

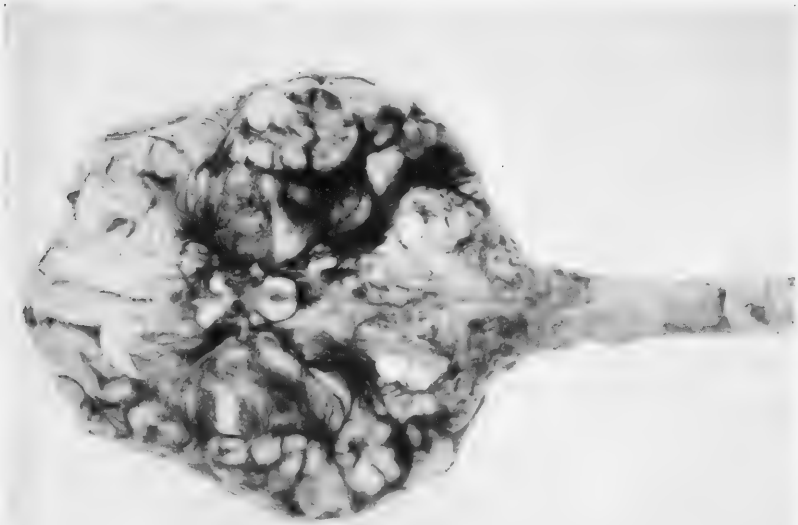
PLATE 28.

FIG. 14. Dog 14. Thalamus. Deep perivascular lesions. Hematoxylin-eosin stain. × 20.

FIG. 15. Same. Perivascular invasion of white matter following thrombosis. Hematoxylin-eosin stain. × 300.

FIG. 16. Dog 15. Early invasion of region of commissures by pneumococci. Gram stain. × 1000. *C* = central canal of cord.

FIG. 17. Pneumococci infiltrating between myelinated nerve fibers just outside a region of purulent central myelitis. Gram stain. × 1000.



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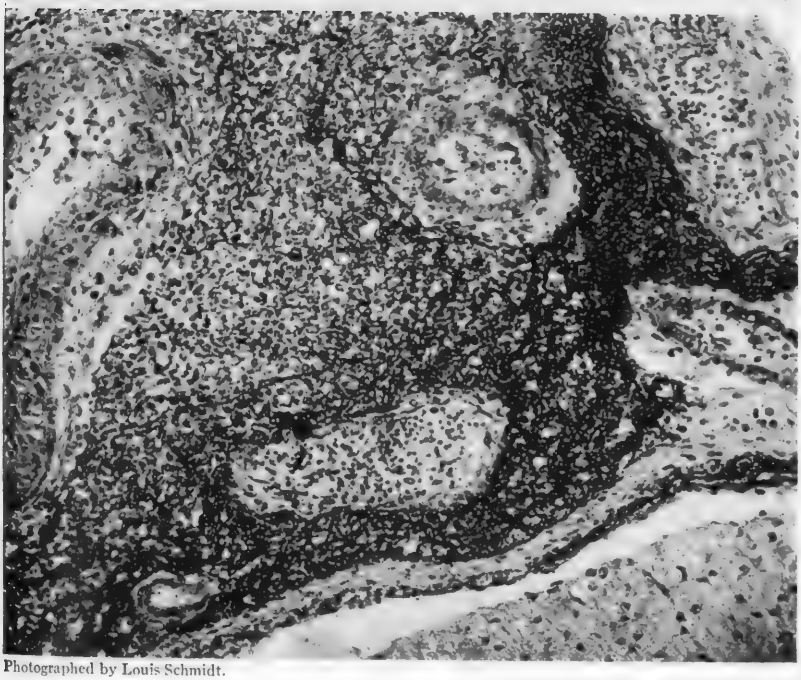

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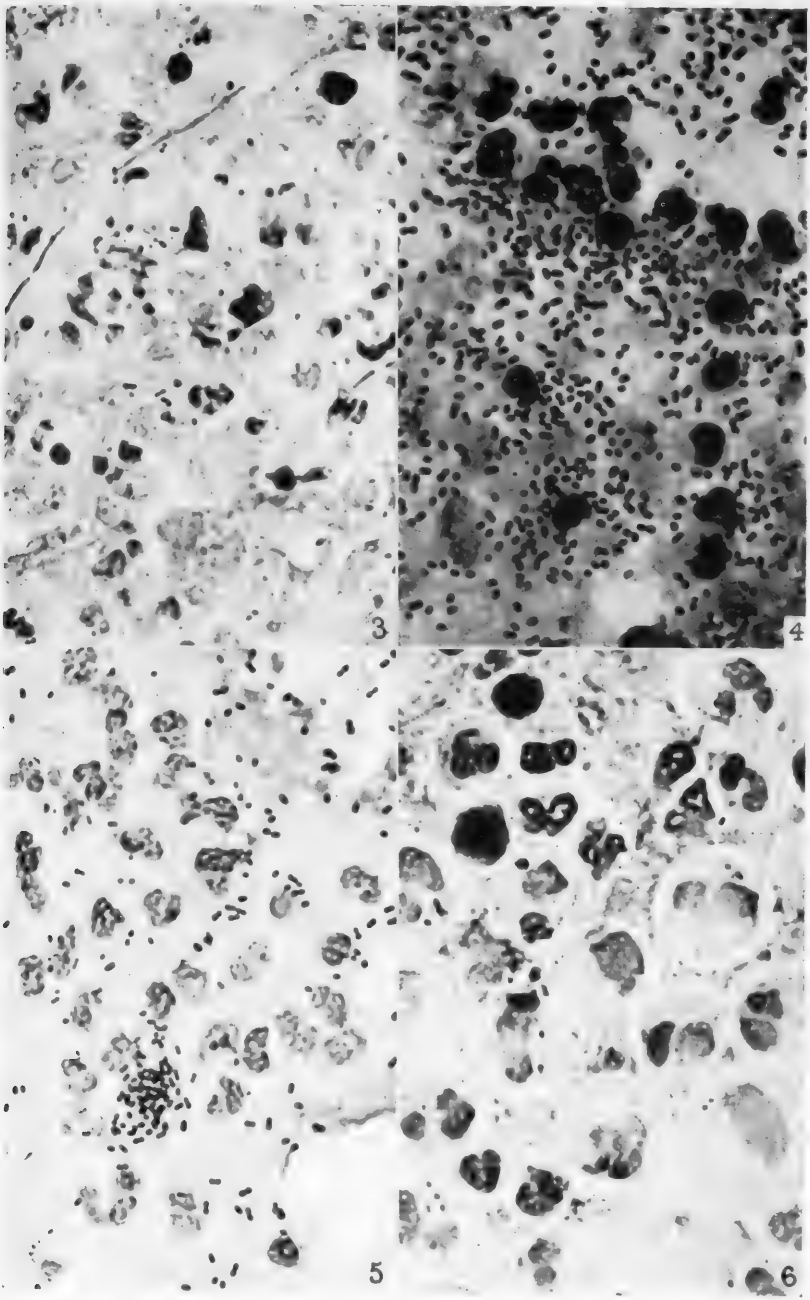

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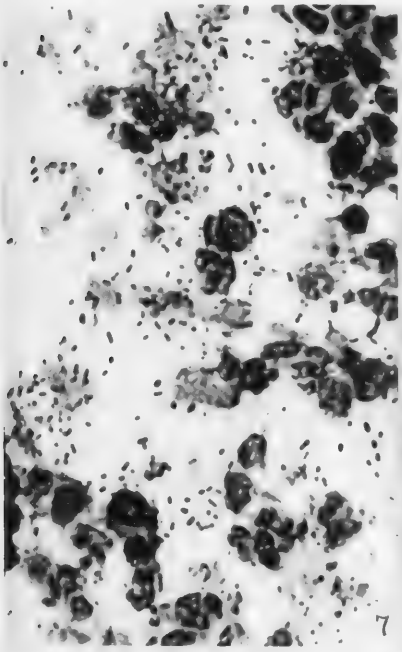

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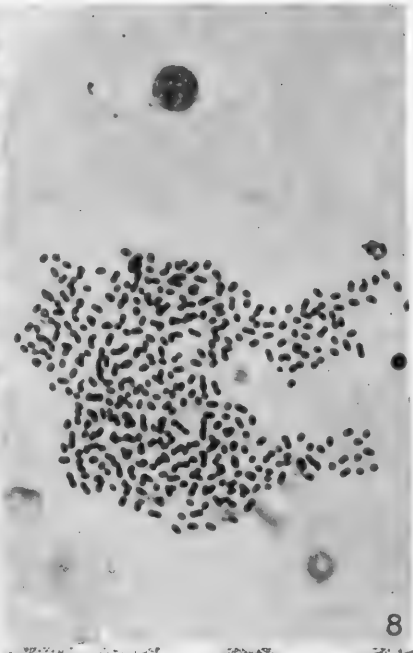

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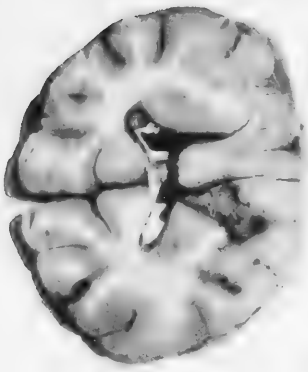

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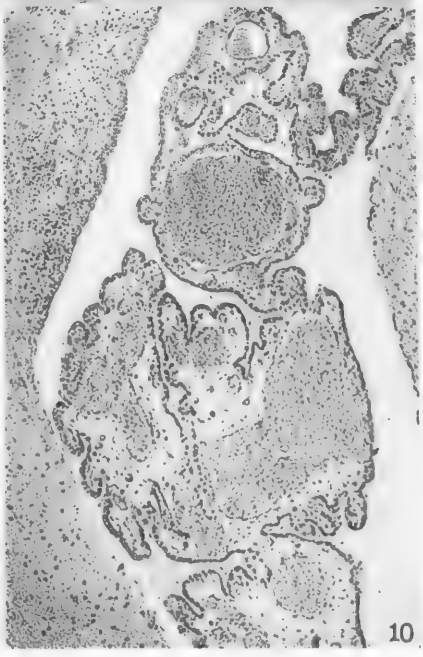

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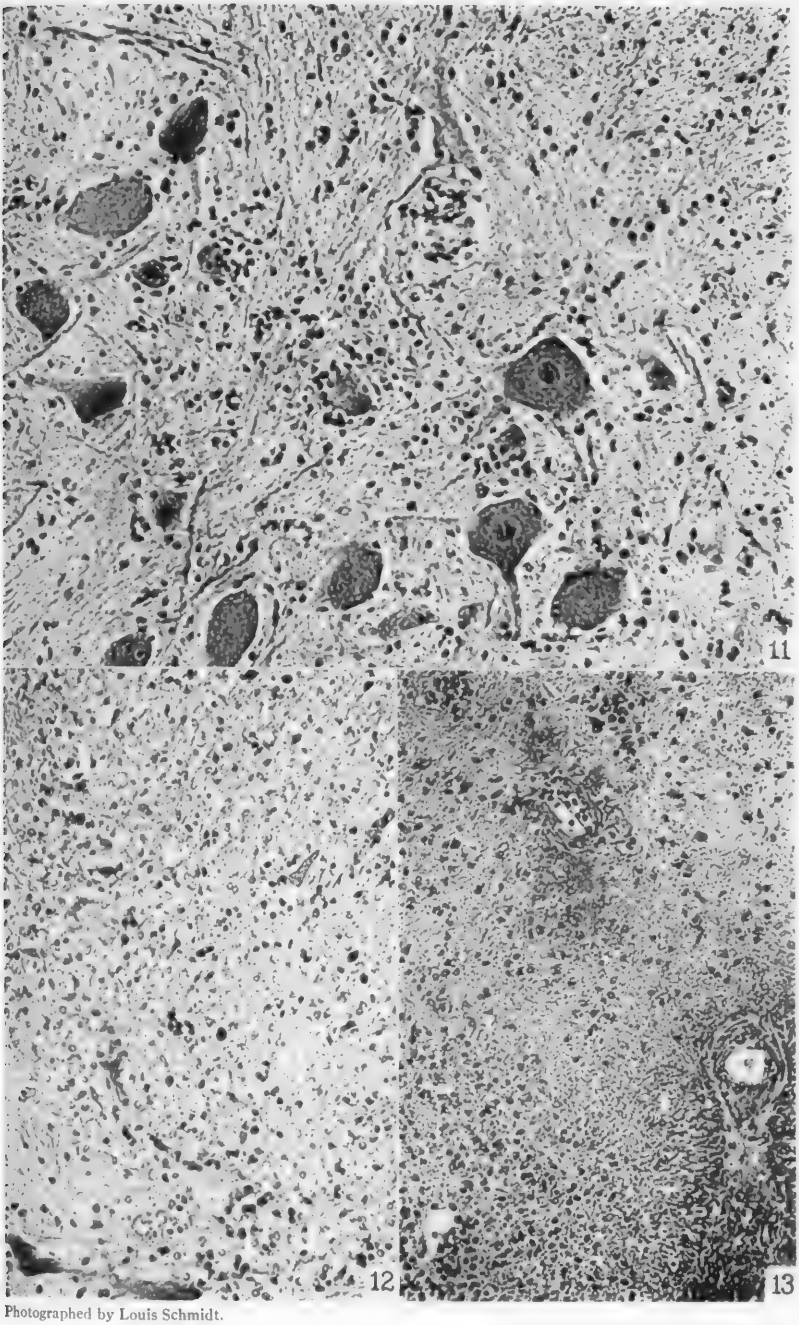

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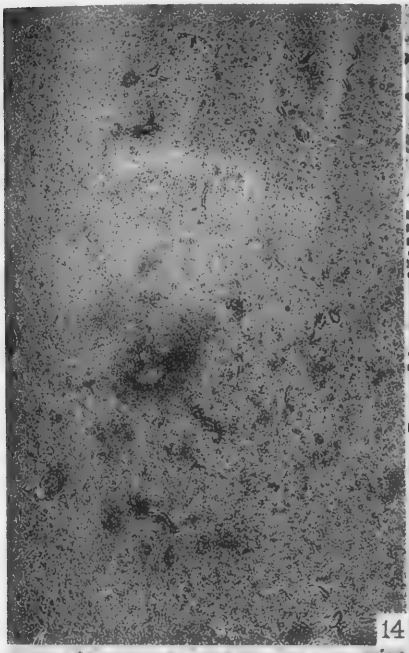

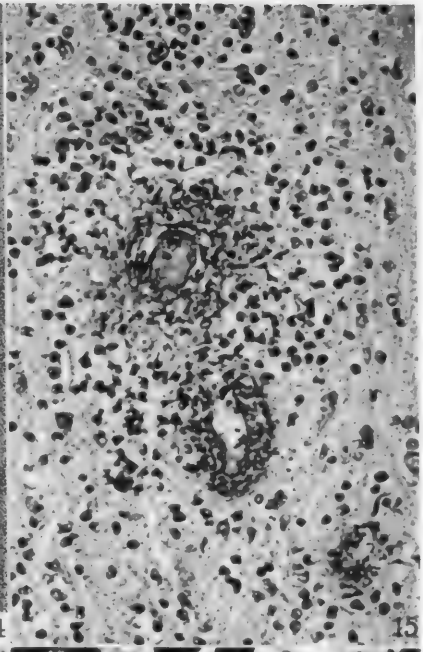

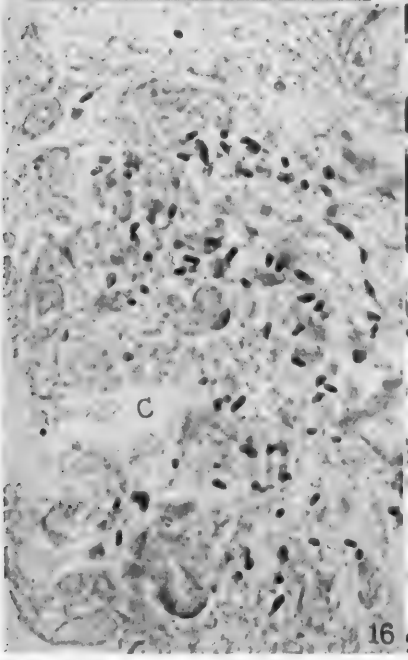

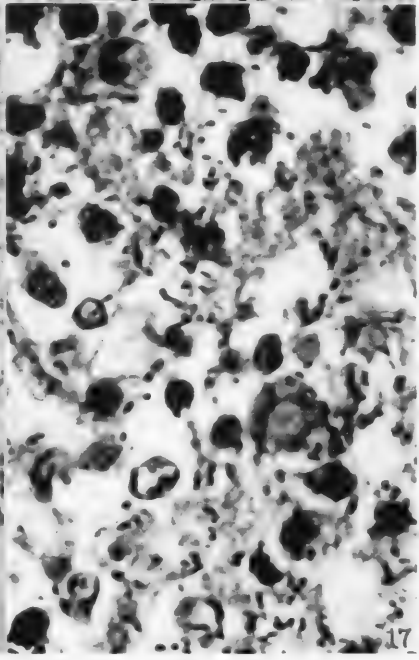

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THE PRODUCTION OF SKIN NECROSIS BY CERTAIN AUTOLYSATES OF PNEUMOCOCCUS (TYPES I AND II).

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Many studies have been made on the various toxic substances obtained from pneumococci. Rosenow (1) and Cole (2) investigated the anaphylactic-like reactions produced in guinea pigs by the intravenous inoculation of autolysates of pneumococci and of the peritoneal exudates of animals dead of pneumococcus septicemia. The hemotoxin of the pneumococcus has been studied by Cole (3), Avery (4), and Neill (5), and the purpura-producing principle of the autolysates by Julianelle and Reimann (6). The effect caused by these various products of the pneumococcus when injected into the skin of man and animals has also been studied (7). No mention, however, has been made of the production of skin necrosis by sterile pneumococcus products, except by Zinsser (8), who was able to produce necrosis in the skin of large normal guinea pigs and in small guinea pigs which had been previously sensitized to the pneumococcus, by the intracutaneous inoculation of certain heat-stabile aerobically produced autolysates of this organism.

When rabbits or guinea pigs are given virulent pneumococci (Type I) intradermally in doses so adjusted that the animals die in 24 to 48 hours, there usually develops at the site of inoculation an area of greenish necrosis several cm. in diameter. Except for its greenish color, the appearance of such a lesion is very similar to that produced by the intracutaneous inoculations of the staphylococcus exotoxin (10), and, therefore, it seemed possible that the pneumococcus lesion might be caused by the same kind of poison. The experiments to be described deal with some investigations made to determine the nature of this necrotizing agent.

EXPERIMENTAL.

The strain of Pneumococcus I used in this work was isolated in November, 1924, from the blood of a patient with lobar pneumonia at the Presbyterian Hospital. Since then it has been passed through rabbits or guinea pigs at least once in every 2 weeks and is now of such virulence that 1–10,000,000 cc. regularly kills rabbits. In mice the lethal dose is 1–1,000,000 cc. No higher dilutions were tried.

Numerous preliminary experiments were conducted to find out whether the necrotizing toxin could be obtained from filtrates of various aerobic and anaerobic broth cultures, exudates, and the expressed fluids of organs and tissues of rabbits and guinea pigs which had died of pneumococcus infection, but the results were uniformly negative.

We next began a study of the autolysates of pneumococci for the presence of this hypothetical necrotizing poison. In general, the method used was to take up the sedimented pneumococci from well centrifuged broth cultures in saline solution, pneumococcus broth, or other fluid, and to allow the suspensions to autolyze, with and without a vaseline seal, at room temperature for different periods of time. It was soon observed that a slight necrotizing action was sometimes obtained with the preparations sealed with vaseline, while the preparations kept without the vaseline seal never possessed this property. From this we inferred that the poison either was formed only in the absence of oxygen, or if formed, was destroyed by oxidation. Various methods of protecting preparations from oxidation were tried, with methylene blue as an indicator. It was found that the addition of sodium hydrosulfite (5), a reducing agent, interfered with the formation of the poison; and that the addition of other reducing agents, such as cysteine (9), live *B. coli*, or yeast, was of no apparent advantage in obtaining an active autolysate. Below I have described in some detail the method which is now being used for the preparation of this toxin.

Preparation of the Necrotizing Poison.

The pneumococci are grown on double strength veal infusion broth containing 4 per cent Witte peptone and 0.5 per cent salt. Flasks are filled nearly to the top with the medium which has been brought to a pH of 7.8. The medium is sterilized in the Arnold sterilizer by the intermittent method.

Each flask containing 200 cc. was inoculated with 1 large loopful of blood from the heart of a guinea pig or rabbit which had died of a pneumococcus infection. After 18 to 24 hours in the incubator, the broth cultures were chilled and then centrifuged at high speed. After centrifuging, they were chilled, the supernatant fluid poured off carefully, and the pneumococci taken up in a quantity of freshly boiled and chilled broth equal to that of the sediment with the small amount of supernatant fluid remaining. For pneumococcus sediment from 100 cc. of broth culture, there should be in all approximately 1.5 cc. of fluid. The pH of the pneumococcus suspensions is brought to 7 or 7.2 and cultures taken to make sure that only pneumococci are present in the preparation. The pneumococcus suspension is distributed into narrow test-tubes, which are then chilled for at least 30 minutes. After this, any bubbles present on the surface of the suspension are gotten rid of with a hot platinum loop and heavy vaseline seals added to all the tubes. The tubes are left at room temperature in the dark at 22–24°C. for 6 to 8 days and then placed in the ice box until used. Immediately before use, the autolysates are centrifuged, iced, the seals opened, and the clear supernatant fluid filtered through a well iced Berkefeld apparatus. This filtrate contains the necrotizing poison. It is necessary to keep the preparations chilled when they are exposed to the air, otherwise they become oxidized and the toxicity disappears.

Young guinea pigs weighing from 250 to 350 gm. were inoculated intradermally on the side with 0.1 cc. of sterile pneumococcus necrotizing poisons. The reaction to the intracutaneous inoculation in these animals is as follows: 10 to 30 minutes after the inoculation, a small purplish area, which gradually increases in size, appears at the site of inoculation. With strong poisons, this area covers a zone of 2 to 3 cm. in diameter within 2 to 4 hours. At this time the area usually appears purplish black, with an encircling border which is bright red in color and which appears to be hemorrhagic. 18 to 24 hours after the inoculation, the central purple zone has become definitely yellowish and necrotic. Several days later, this necrotic area has become a brown scab.

Properties of the Necrotizing Poison.

When filtration of an active autolysate is carried out rapidly through a new N or V Berkefeld filter, with every precaution taken to have the apparatus and autolysate well chilled, there is practically no loss of its activity. Such a filtrate will remain active for several weeks if it is sealed with vaseline immediately after filtration and preserved in the ice box. If opened, even when kept packed in ice, it quickly deteriorates.

The necrosis-producing principle of an autolysate is completely destroyed when heated under vaseline seal at 60°C. for 5 minutes. Under these conditions, with a pH of 6.8 to 7, there is usually a slight clouding of the filtrate.

Are the Pneumococcus Hemotoxin and Necrotizing Poisons Related?

There is no doubt that our necrotizing autolysates contain other pneumococcus products such as the poison which produced the anaphylactic-like symptoms, the hemotoxin, and the purpura-producing principle. Is, then, the necrotizing toxin identical with one of these previously recognized poisonous substances which has been demonstrated by other methods? Apparently, the only toxic product of the pneumococcus which need concern us is the hemotoxin which, as regards thermolability, sensitiveness to oxidation, and ability to be neutralized by immune serum prepared against it, appears to be very similar to the necrosis-producing poison. During the course of this work we have titrated many of our autolysates for the presence of the hemotoxin, and have found, as would be expected, that they always contained more or less of the hemolytic substance, but there was no parallelism between the amount of hemotoxin and of the necrotizing substances present in an autolysate. Moreover, the extracts produced by the freezing and thawing method of Cole and Avery, while rich in hemotoxin, contained little or no necrotic activity. Furthermore, we have been able to separate the hemotoxic and necrotizing substances by treating the autolysate with the red cells of rabbits or guinea pigs. By this procedure the hemotoxin is entirely removed by the red cells, while the necrotizing poison remains unaffected in the supernatant fluid. These last experiments seem to prove conclusively that the hemotoxin and necrotizing poison are different entities. An experiment showing the selective adsorption of the hemotoxin is described below.

This autolysate caused complete hemolysis of 2.5 cc. of a 1 per cent suspension of washed rabbit red cells in a dilution of 1–200 and 50 per cent hemolysis in a dilution of 1–1000. 0.4 cc. of washed rabbit red cells was thoroughly mixed with 2 cc. of chilled necrotizing filtrate. The mixture was allowed to stand for 4 minutes at 2°C. and then centrifuged at high speed for 5 minutes, care being taken to have ice water in the centrifuge cups. After centrifuging, the cold clear slightly green-

ish supernatant fluid was pipetted off and used along with the original filtrate in the tests for hemotoxin. Table I shows the results of this experiment.

That the hemotoxin was removed by a specific combination with the red cells was proved by washing the centrifuged red cells in cold salt solution and resuspending them in warm salt solution. Hemolysis of the red cells took place promptly.

Very recently, by a slightly different method, Neill and Fleming (11) have also been able to completely remove the hemotoxin from pneumococcus extracts by adsorption with red cells.

Antigenic Properties of the Necrosis-Producing Principle.

Three rabbits were injected intracutaneously at weekly intervals with 1 cc. doses of sterile necrotizing filtrates from Pneumococcus I. Six injections were given

TABLE I.

Action of Hemotoxin-Free Autolysate.

Autolysate	Hemotoxic action		Necrotizing action	
	Before adsorption	After adsorption	Before adsorption	After adsorption
0.1 cc.	++++	0	++++	++++
0.02 cc.	++++	0	+	+

in all. The last two inoculations caused no reaction at the sites of injection. The rabbits were bled 10 days after the last inoculation and their serums titrated for the presence of neutralizing antibodies.

These tests were carried out as follows: 0.9 cc. of a well chilled necrotizing filtrate was placed in each of three narrow test-tubes. To the first tube was added 0.1 cc. of the antiserum; to the second tube 0.1 cc. of normal rabbit serum; and to the third the same amount of broth. The contents of the tubes were well mixed, and a heavy vaseline seal was then added to each tube. After standing at room temperature for 1 hour, the tubes were again chilled, the vaseline seals removed, and the preparations in amounts of 0.1 cc. were injected intracutaneously into a small guinea pig. The results are summarized in Table II.

Numerous similar experiments have been made, and it has been found that our immune serum in dilution of 1–10 neutralized the necrotizing poison, whereas normal rabbit serum in the same dilution had no detoxicating effect. The immune serum diluted 1–20 only par-

tially neutralized the toxin. Occasionally slight inactivation occurred with normal serums diluted 1–5.

These experiments seem to show that the immunization of rabbits with necrotizing filtrates causes the production of neutralizing antibodies. Interpretation of these results, however, is complicated by the fact that considerable cloudiness develops in the mixtures of immune serum and toxin, probably due to the action of the antiprotein precipitins, which the antiserums contain, on the pneumococcus protein which is present in the filtrates. From these experiments alone, it would be impossible to say whether the neutralization of the necrotizing poison by this immune serum is really a neutralization, or whether the active substance is merely carried down with the precipitated particles of protein. The same criticism applies to our experiments with Pneumococcus II autolysates as described below.

TABLE II.

Dilution of normal serum	Reaction on guinea pig	Dilution of Type I serum	Reaction on guinea pig
1–5	++	1–5	0
1–10	+++	1–10	0
1–20	+++	1–20	++

Is the Necrosis-Producing Principle Type-Specific or Species-Specific?

It is of considerable interest to know if the necrotizing autolysates prepared from other types of pneumococci can be neutralized by the serums produced against our preparations from Pneumococcus I. To investigate this point, we tested the neutralizing action of our Pneumococcus I antiserum against the necrotizing autolysates prepared from a Pneumococcus II strain which was isolated in October, 1927, from the blood of a patient with pneumonia at the Presbyterian Hospital. It was found that the Type I antinecrotizing serums in dilutions of 1–10 completely neutralized the Type II poison.

As far as they go, these experiments appear to indicate that the necrotizing principles of Pneumococcus I or II are antigenically similar and are probably, therefore, like the pneumococcus hemotoxin, species- and not type-specific.

CONCLUSIONS.

1. Certain pneumococcus autolysates produce necrosis when injected into the skin of guinea pigs.

2. The necrosis-producing principle can be filtered through a Berkefeld N filter, is extremely thermolabile, and is very sensitive to oxidation.

3. The necrotizing poison can be separated from the pneumococcus hemotoxin by adsorption with red cells. This removes the hemotoxin and leaves the necrosis-producing principle unaffected.

4. The necrotizing substances obtained from Pneumococcus Types I and II are neutralized by the antiserum prepared with Pneumococcus I.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

I. Results of Repeated and Prolonged Observations on Normal Rabbits.

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A study of calcium and inorganic phosphorus in the blood of rabbits was undertaken by us as a phase of a more comprehensive investigation of the problem of constitutional factors in relation to the occurrence and course of disease, with especial reference to the influence of environmental conditions (1). In extending this investigation to chemical constituents of the blood, it seemed desirable to begin with substances which are supposed to maintain some kind of equilibrium and for which quantitative methods have been developed that would permit of a systematic study in large series of animals or over long periods of time.

Calcium and inorganic phosphorus appeared to meet these fundamental requirements. Moreover, the work of Grant and Gates (2) indicated that the amounts of these substances in the blood of rabbits are subject to spontaneous (seasonal) variation, and hence, that they may be affected by environmental conditions—a supposition that is also supported by recent studies of rickets. There was the additional possibility that a relation might be found between the variations in calcium and inorganic phosphorus and the susceptibility of animals to disease, since organs concerned in regulating the concentration of these substances in the blood frequently show marked changes in the reaction to disease. This is especially true of the two diseases (syphilis and malignant disease) which have been used as the basis of our study of the influence of constitutional factors.

The experiments that have been carried out are divisible into two

groups: first, those dealing with conditions that obtain in normal rabbits, and, second, those concerned with animals inoculated with *Treponema pallidum* or a malignant tumor. The results obtained for normal animals will be reported first. The present paper will be limited to a consideration of mean normal values, and deviations from such values for calcium and inorganic phosphorus in the blood of rabbits as determined by repeated examinations of 4 groups of animals over periods of from 16 to 32 weeks. In subsequent papers, these basic results will be compared with other results from the point of view of the various factors inherent in the animal material or of an environmental (experimental) nature which may affect the calcium and inorganic phosphorus content of the blood in a given case.

Material and Methods.

There were several important considerations which governed the conduct of these experiments. In the first place, an effort was made to obtain a fair sample of the animal material available for experimental purposes, and the animals were caged and cared for in the usual manner. In the second place, the period of observation and the interval at which blood examinations were made were determined with reference to other experiments, while groups of animals were distributed so as to cover as long a period of time as possible.

The results reported below are based on examinations of 4 groups of male rabbits, most of which were 6 to 8 months old at the beginning of the experiments; a few were older, and a few others may have been a month or 6 weeks younger. The period covered by the investigation was from October 8, 1926, to July 1, 1927, inclusive. While under observation, the animals were caged separately and kept in well lighted, well ventilated rooms which were heated during the colder weather. The diet consisted of hay, oats and cabbage.

Group I.—10 rabbits, 5 white and 5 black. Between October 8, 1926, and May 18, 1927, the blood of these animals was examined at weekly or biweekly intervals.

Group II.—5 rabbits, gray, brown or black. These animals came from the same stocks as those of Group I and were placed under observation at the same time, but no blood examinations were made until December 1, 1926, or until they had been caged and under observation for approximately 2 months. Blood analyses on these animals were made at somewhat irregular intervals, but, for the most part, they paralleled those of Group I. The period covered was from December 1, 1926, to June 8, 1927.

Group III.—10 rabbits, grays and browns. These animals were slightly younger than those of Groups I and II. Biweekly examinations were made from January 14 to July 1, 1927.

Group IV.—11 rabbits, browns and grays. Most of these animals were still

younger than those of Group III. From March 11 to May 13 examinations were made at weekly intervals and biweekly from May 13 to July 1, 1927.

The observations made on the 4 groups of rabbits may be summarized as follows:

Group	Observation period	Group determinations	Individual determinations
I	Oct. 8 to May 18	22	216
II	Dec. 1 to June 8	17	85
III	Jan. 14 to July 1	15	143
IV	Mar. 11 to July 1	15	153
Combined	Oct. 8 to July 1	69	597

Determinations of calcium and inorganic phosphorus were made on the blood serum according to the methods of Kramer and Tisdall (3) and of Tisdall (4) respectively.

At first, the procedure followed was somewhat irregular with respect to the disposition of the blood after it was drawn and the interval of time between bleeding and analysis. In some instances, the blood was permitted to stand at room temperature for several hours, or until there was a well defined separation of serum and clot, and then transferred to the ice box until the following morning when the analyses were made. It thus happened that an interval of 12 to 18 hours elapsed between bleeding and analysis.

After the first few weeks, however, a uniform procedure was adopted. On the day preceding examination, animals were fed as usual in the afternoon and were bled early the following morning before they had received any additional food. 8 cc. of blood was drawn from a marginal ear vein into a graduated centrifuge tube and permitted to clot at room temperature. The clot was separated from the wall of the tube and as soon as sufficient serum was expressed, the blood was centrifuged and analysis commenced at once. The time between the bleeding and analysis was thus reduced to 1 to 2 hours, and the use of the ice box was eliminated.

In order to expedite the work, and as a precautionary measure in the case of syphilitic animals, the serum was measured with a 1 cc. tuberculin syringe graduated to 0.01 cc. instead of a pipette. The syringes were fitted with 18–20 gauge needles and their accuracy was carefully controlled. As far as possible, the work was so arranged that a given procedure was always performed by the same person; all titrations and all colorimetric readings were made by one person (Howard).

The results given represent determinations of calcium and phosphorus on the same samples of blood. Numerous determinations were made in duplicate to control the experimental error in the methods as carried out. In such cases, the mean value of the determinations was recorded and used as a single determination in the results to be reported. The mean error for duplicate determinations of

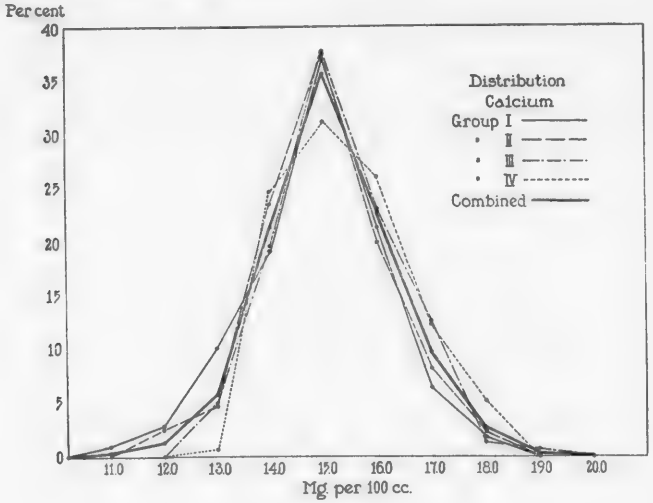
calcium and inorganic phosphorus was approximately 0.2 mg. per 100 cc. of serum, or about 1.25 per cent and 4.50 per cent of the respective mean values.

In addition to the primary values for calcium and inorganic phosphorus, we have computed values for the sum and product of the two substances, the ratio of calcium to inorganic phosphorus, and of the product to the sum, and for the sum of these two ratios.

The results are presented in the form of tabulated summaries (Tables I to XV) and a series of text-figures (Text-figs. 1 to 7). Values are given in Tables I to XV for individual groups of animals and for the 4 groups combined. The combined values do not represent means, but are obtained by actual combination of all observations. It will be noted that a few of the higher values given in the distribution tables are not recorded in the text-figures.

RESULTS.

As the purpose of this paper is to give a general idea of the calcium and inorganic phosphorus content of rabbits' blood, under certain prescribed conditions, it seems unnecessary to record the details of individual examinations. The results are reported, therefore, in the form of tabulated summaries (Tables I to XV) supplemented by a series of graphs (Text-figs. 1 to 7).



TEXT-FIG. 1.

TABLE I.

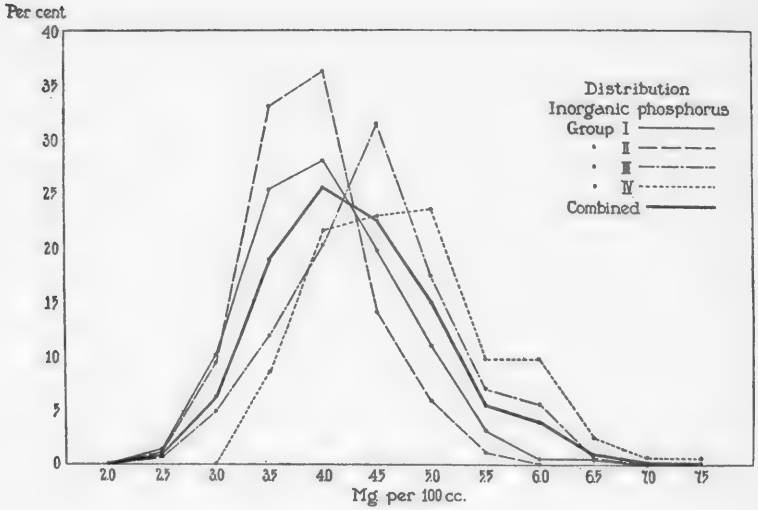
Calcium.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
		mg. per 100 cc.	*mg.*	*mg.*	*mg.*	*per cent*
I	219	15.3±0.06	11.1	21.2	1.30	8.50
II	85	15.5±0.10	12.6	22.8	1.38	8.90
III	143	15.7±0.07	13.4	19.7	1.17	7.45
IV	154	15.8±0.06	13.8	18.9	1.12	7.09
Combined	601	15.6±0.03	11.1	22.8	1.25	8.01

TABLE II.

Distribution of Values for Calcium.

Mg. per 100 cc.	Group I	Group II	Group III	Group IV	Combined
11.0–11.4	1				1
11.5–11.9	1				1
12.0–12.4					
12.5–12.9	6	2			8
13.0–13.4	10	1	2		13
13.5–13.9	12	3	5	1	21
14.0–14.4	18	7	11	17	53
14.5–14.9	24	13	17	21	75
15.0–15.4	40	25	32	28	125
15.5–15.9	41	7	22	20	90
16.0–16.4	35	9	21	25	90
16.5–16.9	12	8	12	15	47
17.0–17.4	10	5	7	12	34
17.5–17.9	4	2	11	7	24
18.0–18.4	2	1		6	9
18.5–18.9	2	1	2	2	7
19.0–19.4					
19.5–19.9			1		1
20.0–20.4					
20.5–20.9					
21.0–21.4	1				1
21.5–21.9					
22.0–22.4					
22.5–22.9		1			1
Observations	219	85	143	154	601


Per cent
40
35
30
25
20
15
10
5
0
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Mg per 100 cc.
Distribution
Inorganic phosphorus
Group I
II
III
IV
Combined

TEXT-FIG. 2.

TABLE III.

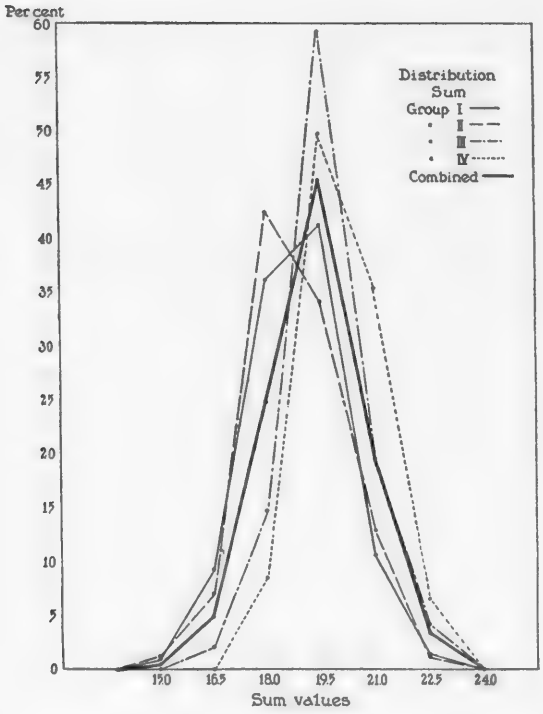
Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
		mg. per 100 cc.	*mg.*	*mg.*	*mg.*	*per cent*
I	216	4.25±0.03	2.60	6.74	0.68	16.00
II	85	4.11±0.04	2.95	5.77	0.51	12.41
III	143	4.67±0.04	2.64	6.76	0.75	16.06
IV	153	4.96±0.04	3.59	7.50	0.80	16.13
Combined	597	4.51±0.02	2.60	7.50	0.78	17.29

TABLE IV.

Distribution of Values for Inorganic Phosphorus.

Mg. per 100 cc.	Group I	Group II	Group III	Group IV	Combined
2.50–2.74	2		1		3
2.75–2.99	1	1			2
3.00–3.24	7		2		9
3.25–3.49	15	8	5		28
3.50–3.74	18	13	10	5	46
3.75–3.99	37	15	7	8	67
4.00–4.24	33	17	11	17	78
4.25–4.49	27	13	18	16	74
4.50–4.74	27	9	29	25	90
4.75–4.99	16	3	16	10	45
5.00–5.24	15	4	13	22	54
5.25–5.49	9	1	12	14	36
5.50–5.74	4		6	11	21
5.75–5.99	3	1	4	4	12
6.00–6.24			8	8	16
6.25–6.49	1			7	8
6.50–6.74	1			3	4
6.75–6.99			1	1	2
7.00–7.24				1	1
7.25–7.49					
7.50–7.74				1	1
Observations	216	85	143	153	597


Per cent
60
55
50
45
40
35
30
25
20
15
10
5
0
Distribution
Sum
Group I
II
III
IV
Combined
150
165
180
195
210
225
240
Sum values

TEXT-FIG. 3.

TABLE V.

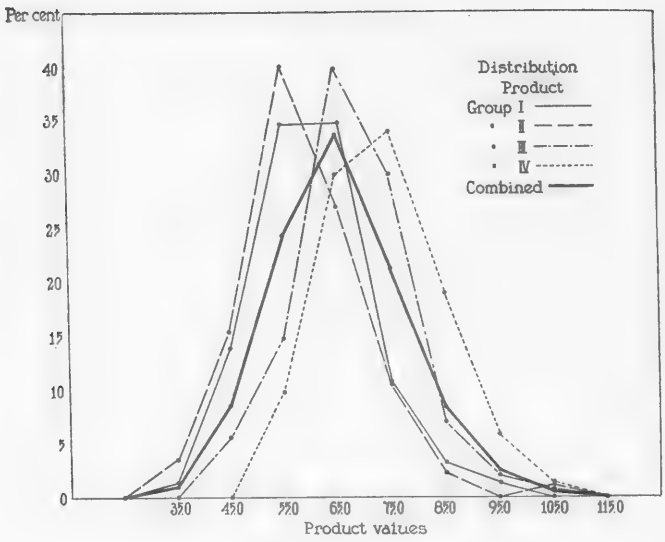
Sum of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						per cent
I	216	19.6±0.06	15.51	25.81	1.31	6.68
II	85	19.7±0.11	16.40	27.50	1.53	7.77
III	143	20.4±0.06	17.50	23.90	1.11	5.44
IV	153	20.8±0.06	18.30	23.60	1.01	4.86
Combined	597	20.1±0.04	15.51	27.50	1.29	6.42

TABLE VI.

Distribution of Values for the Sum of Calcium and Inorganic Phosphorus.

Sum values	Group I	Group II	Group III	Group IV	Combined
15.5–15.9	1				1
16.0–16.4	1	1			2
16.5–16.9	2	2			4
17.0–17.4	4	1			5
17.5–17.9	14	3	3		20
18.0–18.4	14	7		2	23
18.5–18.9	24	11	5	5	45
19.0–19.4	40	18	16	6	80
19.5–19.9	34	8	31	15	88
20.0–20.4	32	13	29	29	103
20.5–20.9	23	8	25	32	88
21.0–21.4	11	4	12	23	50
21.5–21.9	7	6	11	21	45
22.0–22.4	5	1	5	10	21
22.5–22.9	3	1	2	7	13
23.0–23.4			1	2	3
23.5–23.9			3	1	4
24.0–24.4					
25.5–25.9	1				1
27.5–27.9		1			1
Observations	216	85	143	153	597


Per cent
40
35
30
25
20
15
10
5
0
Distribution
Product
Group I
" II
" III
" IV
Combined
35.0
45.0
55.0
65.0
75.0
85.0
95.0
105.0
115.0
Product values

TEXT-FIG. 4.

TABLE VII.

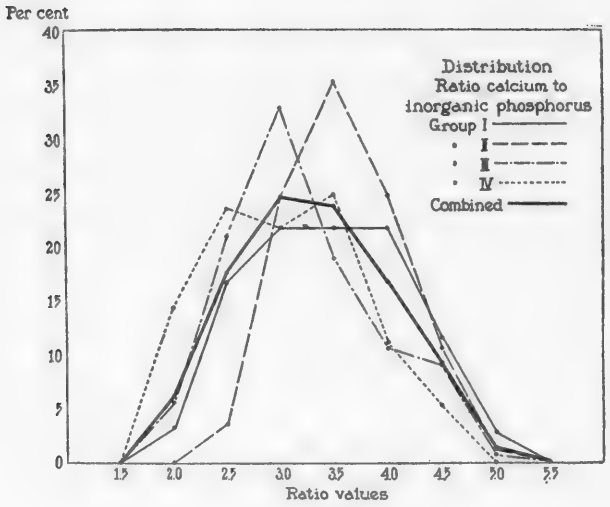
Product of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						per cent
I	216	65.3±0.5	40.3	99.8	10.4	15.93
II	85	64.0±0.8	43.5	108.1	10.6	16.56
III	143	73.0±0.6	45.2	107.9	10.8	14.79
IV	153	78.1±0.6	55.9	113.9	10.8	13.83
Combined	597	70.2±0.3	40.3	113.9	12.0	17.09

TABLE VIII.

Distribution of Values for the Product of Calcium and Inorganic Phosphorus.

Product values	Group I	Group II	Group III	Group IV	Combined
40– 44	3	3			6
45– 49	10		2		12
50– 54	20	13	6		39
55– 59	38	19	10	4	71
60– 64	37	15	11	11	74
65– 69	44	15	22	20	101
70– 74	31	8	35	26	100
75– 79	15	6	22	30	73
80– 84	8	3	21	22	54
85– 89	5	2	7	18	32
90– 94	2		3	11	16
95– 99	3		1	7	11
100–104			2	2	4
105–109		1	1	1	3
110–114				1	1
Observations	216	85	143	153	597


Per cent
40
35
30
25
20
15
10
5
0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
Ratio values
Distribution
Ratio calcium to
inorganic phosphorus
Group I
I
II
IV
Combined

TEXT-FIG. 5.

TABLE IX.

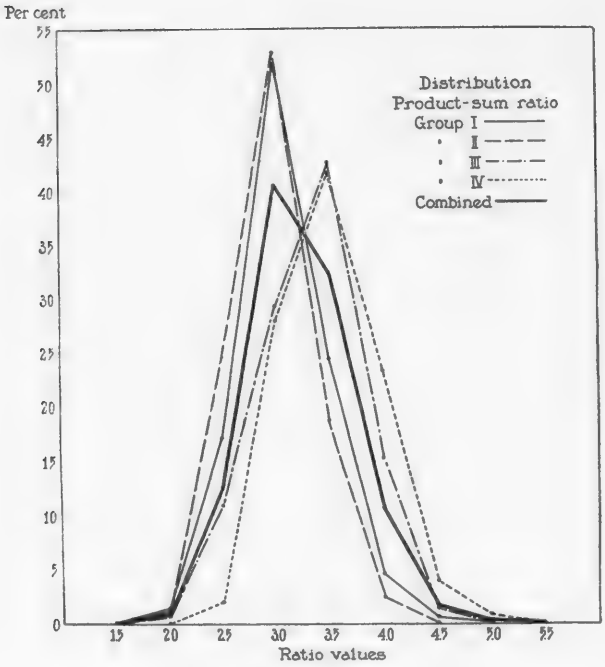
Ratio of Calcium to Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						per cent
I	216	3.70±0.03	2.16	6.37	0.73	19.73
II	85	3.83±0.04	2.66	5.00	0.53	13.84
III	143	3.47±0.04	2.19	6.48	0.74	21.33
IV	153	3.30±0.04	2.03	4.71	0.65	19.70
Combined	597	3.57±0.02	2.03	6.48	0.72	20.17

TABLE X.

Distribution of Values for the Ratio of Calcium to Inorganic Phosphorus.

Ratio values	Group I	Group II	Group III	Group IV	Combined
2.00–2.24	2		1	6	9
2.25–2.49	5		7	16	28
2.50–2.74	15	1	16	11	43
2.75–2.99	21	2	14	25	62
3.00–3.24	23	7	21	23	74
3.25–3.49	24	14	26	9	73
3.50–3.74	22	11	18	23	74
3.75–3.99	25	19	9	15	68
4.00–4.24	26	13	7	16	62
4.25–4.49	21	8	8	1	38
4.50–4.74	17	3	8	8	36
4.75–4.99	8	6	5		19
5.00–5.24	3	1			4
5.25–5.49	3		1		4
5.50–5.74			1		1
5.75–5.99					
6.00–6.24					
6.25–6.49	1		1		2
Observations	216	85	143	153	597


Per cent
55
50
45
40
35
30
25
20
15
10
5
0
Distribution
Product-sum ratio
Group I
I
III
IV
Combined
15
20
25
30
35
40
45
50
55
Ratio values

TEXT-FIG. 6.

TABLE XI.

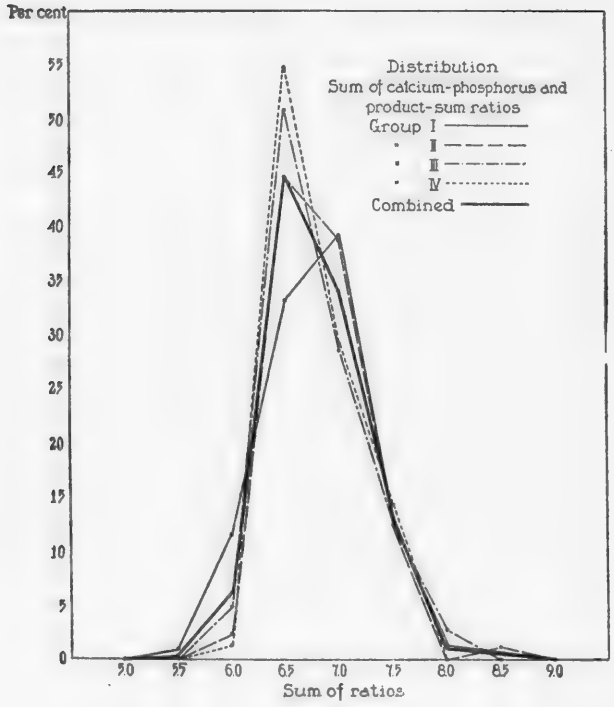
Ratio of Product to the Sum of Calcium and Inorganic Phosphorus.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						per cent
I	216	3.32±0.02	2.25	4.63	0.39	11.75
II	85	3.24±0.02	2.46	4.19	0.33	10.19
III	143	3.58±0.02	2.29	4.68	0.40	11.17
IV	153	3.75±0.02	2.96	5.02	0.42	11.20
Combined	597	3.47±0.01	2.25	5.02	0.44	12.68

TABLE XII.

Distribution of Values for the Ratio of the Product to the Sum of Calcium and Inorganic Phosphorus.

Ratio values	Group I	Group II	Group III	Group IV	Combined
2.25–2.49	3	1	1		5
2.50–2.74	13	4	3		20
2.75–2.99	24	17	12	3	56
3.00–3.24	54	27	11	16	108
3.25–3.49	58	18	31	27	134
3.50–3.74	31	11	40	33	115
3.75–3.99	22	5	21	31	79
4.00–4.24	8	2	16	21	47
4.25–4.49	2		6	15	23
4.50–4.74	1		2	5	8
4.75–4.99				1	1
5.00–5.24				1	1
Observations	216	85	143	153	597


Per cent
55
50
45
40
35
30
25
20
15
10
5
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Sum of ratios
Distribution
Sum of calcium-phosphorus and product-sum ratios
Group I
" II
" III
" IV
Combined

TEXT-FIG. 7.

TABLE XIII.

Sum of the Ratios of Calcium to Inorganic Phosphorus and of the Product to the Sum.

Group	Number of observations	Mean	Minimum	Maximum	Standard deviation	Coefficient of variation
						per cent
I	216	7.02±0.02	5.68	8.62	0.45	6.41
II	85	7.07±0.03	6.11	8.74	0.41	5.80
III	143	7.05±0.02	6.32	8.77	0.41	5.82
IV	153	7.05±0.02	6.49	7.85	0.33	4.68
Combined	597	7.04±0.01	5.68	8.77	0.40	5.68

TABLE XIV.

Distribution of Values for the Sum of the Ratios of Calcium to Inorganic Phosphorus and of the Product to the Sum of the Two Substances.

Sum values	Group I	Group II	Group III	Group IV	Combined
5.50–5.74	1				1
5.75–5.99	1				1
6.00–6.24	10	2			12
6.25–6.49	15		7	2	24
6.50–6.74	32	12	29	32	105
6.75–6.99	40	26	44	52	162
7.00–7.24	54	20	21	25	121
7.25–7.49	31	13	20	20	83
7.50–7.74	20	7	15	18	60
7.75–7.99	8	4	3	4	19
8.00–8.24	2		2		4
8.25–8.49	1		1		2
8.50–8.74	1	1			2
8.75–8.99			1		1
Observations	216	85	143	153	597

TABLE XV.

Summary of Results.

Group	Number of animals	Number of observations	Observation period	Calcium	Inorganic phosphorus	Sum of calcium and phosphorus	Product of calcium and phosphorus	Ratio of calcium to phosphorus	Ratio of product to sum	Sum of two ratios
			wks.	*mg. per 100 cc.*	*mg. per 100 cc.*					
I	10	216	32	15.3	4.25	19.6	65.3	3.70	3.32	7.02
II	5	85	27	15.5	4.11	19.7	64.0	3.83	3.24	7.07
III	10	143	25	15.7	4.67	20.4	73.0	3.47	3.58	7.05
IV	11	153	16	15.8	4.96	20.8	78.1	3.30	3.75	7.05
Combined	36	597	37.5	15.6	4.51	20.1	70.2	3.57	3.47	7.04

DISCUSSION AND CONCLUSIONS.

It will be seen at once that the mean values obtained for calcium are higher than most of those recorded in the literature, and that the values for inorganic phosphorus are perhaps lower. It is well to bear in mind, however, that the significance that may be attached to any series of determinations of calcium and inorganic phosphorus in the blood of animals depends largely upon the conditions under which the determinations are made. As is well known, there are many factors that may affect the values obtained, including inherent differences in the animal material and the method of analysis used as well as the particular procedure employed in carrying out a given method. When all other conditions are uniform, irregularities in the handling of the blood after it is drawn will give rise to surprisingly large differences in the results for both calcium and inorganic phosphorus, as permitting blood to stand tends to decrease calcium values and to increase those for inorganic phosphorus.

It seems desirable, therefore, to emphasize the fact that the results recorded above are to be viewed as results obtained under certain definitely prescribed conditions which differ in several important respects from those governing determinations made by other workers in this field. Moreover, it is to be noted that the conditions varied to some extent with each of the 4 groups of animals comprising this

series. For example, there was a small but definite age difference. The animals of Groups I and II were older and more mature than those of Groups III and IV at the beginning of the experiments, and this initial difference was increased by the extension of the experiments on Groups I and II over a longer period of time, so that the observations made on these animals not only included data for a more advanced age, but represented a mean age considerably above that of the observations made on the animals of Groups III and IV. There was a similar difference of experimental conditions between Groups III and IV, while the observations on Group II differed from those on Group I in that no blood analyses were made on the animals of Group II for 2 months after they were placed under observation.

These particular features of the experiments are mentioned because an examination of the text-figures will show that a line of cleavage between Groups I and II on the one hand, and III and IV on the other, is traceable through all of the distribution curves and to some extent in the tabulated results. With the combined values as the axis of distribution, Groups I and II invariably hang together, or swing to one side, while Groups III and IV swing to the other. Moreover, the extreme positions are usually represented by Groups II and IV.

Whether these peculiarities of the results are in reality attributable to the conditions mentioned or to some other cause, such as the length of cage life (2), or the particular period covered by the observations, the suggested relation is sufficient to indicate the extent to which even slight differences in experimental conditions may affect the results obtained for blood calcium and inorganic phosphorus.

The values obtained for calcium may be regarded as showing a fairly close agreement (Tables I and II and Text-fig. 1). The extreme difference between the means for the 4 groups of animals is only 0.5 mg. or approximately 3.00 per cent of the mean for the combined groups. Still, the small absolute difference between the means for Groups I and IV is nearly 6 times its probable error and, hence, cannot be disregarded.

The most important feature of these results is, however, the range of normal variation. The distribution curves (Text-fig. 1) show a remarkably close agreement in the frequency with which values of a

given magnitude occurred and an unusually symmetrical distribution of all values. The coefficients of variation are comparatively small (7.09 to 8.9 per cent), but values anywhere between 14.0 and 16.0 mg. of calcium per 100 cc. of serum occurred with great frequency, while figures as low as 13.5 or as high as 17.5 mg. (Table II) were by no means rare; and the extreme limits of observation indicate a potential difference in the calcium content of the blood of normal rabbits of as much as 100 per cent.

Inorganic phosphorus was found to be subject to much wider variation than calcium (Tables III and IV and Text-fig. 2). The coefficient of variation is approximately twice that for calcium (17.29 and 8.01 respectively), while the group means for phosphorus show a difference of 0.85 mg. per 100 cc. of serum. This difference is small in absolute value, but is nearly 20.0 per cent of the mean for all groups and is 15 times its probable error. It is safe to assume, therefore, that the values obtained indicate an actual difference in the inorganic phosphorus in the blood of the several groups of animals.

This conclusion is borne out by the distribution frequencies (Table IV and Text-fig. 2) which show that the values obtained for Groups I and II lie at a distinctly lower level than those for Groups III and IV; the difference between modal classes is, in fact, of the same order as that shown by the means.

The limits of probable variation as determined by the standard deviation of the combined results are 3.73 and 5.29 mg. per 100 cc. of serum, but one-third of all values lie outside of these limits, while the extreme limits of normal are sufficiently wide to include values that may differ by as much as 200.0 or even 300.0 per cent.

From the values obtained for calcium and inorganic phosphorus, the relation existing between the two substances may be measured in a number of ways. The ratio of the calcium to the phosphorus and the product of the amounts of the two substances have received the greatest attention. In addition to these values, we have computed values for the sum and for the ratio of the product to the sum, and also for the sum of the calcium-phophorus ratio and the product-sum ratio.

The value for the sum of the calcium and inorganic phosphorus in the serum is determined largely by the calcium, but as it is also affected by the phosphorus, one might expect that the constancy of the value

as compared with that of calcium would be diminished unless the variations in the two substances were so related as to neutralize each other. As is well known, there is an apparent tendency in this direction and in these experiments it was found that on the whole the values for the sum showed less variation (coefficients 6.42 and 8.01 per cent) and were more uniformly distributed than those for calcium (Tables V and VI and Text-fig. 3). It is true that differences between groups were distinctly greater than in the case of calcium, but the agreement is sufficiently close to give evidence of a tendency to the maintenance of an inverse relation between serum calcium and inorganic phosphorus.

Values for the product of calcium and inorganic phosphorus emphasize the phosphorus factor rather than the calcium, reversing the conditions that obtain in the case of the sum. A consideration of the product values given in Tables VII and VIII and Text-fig. 4 show that, while the order of variation is essentially the same as that of inorganic phosphorus (coefficients 17.09 and 17.29 per cent respectively), the distribution of values is more uniform. This may be attributable to the occurrence of coordinate variations in calcium and inorganic phosphorus.

The situation presented by the values obtained for the ratio of calcium to inorganic phosphorus is somewhat surprising in that the ratio between the two substances proves to be less constant than the absolute amounts of either substance (Tables IX and X and Text-fig. 5). There are considerable differences between the standard values for individual groups of animals, and the distribution frequencies are inclined to be irregular. Moreover, all groups show a large standard deviation and correspondingly high coefficients of variation, but combining the results for the 4 groups of animals gives a fairly uniform and symmetrical distribution, a striking feature of which is the high frequency with which values occur over the entire range of standard variation, that is, from ratios of 2.85 to 4.29.

It thus appears that, despite the evidence of a tendency to the observance of an inverse relation between the calcium and inorganic phosphorus in the blood, the ratio of one substance to the other is by no means constant.

By using the product and the sum as a basis of expressing the relation between calcium and inorganic phosphorus, the form of the relation is

reversed, that is, an increase in calcium or a reduction in phosphorus diminishes the ratio value, whereas under the same circumstances, the direct ratio of calcium to phosphorus is increased. Objection may be made to this method of treatment, but the fact remains that by compounding the terms of the ratio in this manner, some interesting points are brought out (Tables XI and XII and Text-fig. 6).

In the first place, the mean values for the two ratios are of a comparable order, but the values obtained for the product-sum ratio of different groups of animals show a closer agreement than those for the calcium-phosphorus ratio. Moreover, the distribution of values is comparatively uniform throughout, while the variability of individual groups and of the series as a whole is less than two-thirds of that found for the calcium-phosphorus ratio.

Finally, known differences in the experimental conditions represented by the 4 groups of animals are expressed with remarkable clarity (Text-fig. 6) by this treatment of ratio values which suggests that the method may be used to advantage in the analysis of results of this kind.

By comparing the group values for the calcium-phosphorus and product-sum ratios, it will be seen that they are not only of a comparable order of magnitude (3.57 and 3.47 for the combined groups), but that a high value of one tends to be associated with a correspondingly low value of the other, so that the mean of the two ratios remains fairly constant at about 3.50, or their sum is approximately 7.00 (Tables XIII and XIV and Text-fig. 7).

The constancy of this relation does not hold for individual observations or for all classes of animals or experimental conditions. With grouped material, the absolute value of the sum or mean of the two ratios may increase or decrease, just as other values do, but tends to preserve a greater degree of constancy under a given set of conditions and appears to be less affected by slight changes in experimental conditions or by experimental errors. It is not certain whether the constancy of this value is due entirely to weighting, or the compounding of terms, or is indicative of the maintenance of a relation between calcium and inorganic phosphorus which is expressed in the maintenance of an equilibrium between the two ratios.

The values for calcium and inorganic phosphorus, as recorded above,

may be regarded as a cross-section of results obtained from a fair sample of laboratory material, and in this sense, may be used as standards of comparison in estimating the probable significance of other determinations. It has been clearly shown, however, that even slight differences in experimental conditions are apt to be reflected in the results obtained. It is evident, therefore, that as experimental conditions are changed, corresponding differences in results may be expected.

It has also been shown that neither the calcium nor the inorganic phosphorus of rabbits' blood can be regarded as constant. As is well known, inorganic phosphorus is the more labile of the two substances and is subject to much greater variation than calcium, but the upper and lower limits of both are far apart. In fact, the range of so called normal variation, or even the range of high frequency variation, is in all probability sufficiently wide to include many variations that occur under pathological as well as physiological conditions.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of 4 groups of normal rabbits at intervals of 1 to 2 weeks over periods of 4 to 8 months. From the data thus obtained, values were calculated for calcium and inorganic phosphorus, and for various relations between the two substances.

It was found that both calcium and inorganic phosphorus showed wide ranges of variation with a tendency to vary in an inverse direction. Still, the relation between the two substances, as expressed by a direct ratio or by the product of the two substances, was less constant than the absolute amounts of either calcium or inorganic phosphorus. The ratio of the product to the sum of the two substances was more constant, and this ratio varied inversely with the ratio of calcium to inorganic phosphorus, so that the mean or the sum of the two values varied comparatively little under a given set of experimental conditions.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

II. Results of Single Determinations on Normal Rabbits from Recently Acquired Stocks.

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Results of a large series of determinations of calcium and inorganic phosphorus in the blood of normal rabbits were reported in a previous paper (1). These results were based on repeated examination of the blood of the same animals, continued over periods of 4 to 8 months. A parallel series of blood analyses was made on small groups of animals within a few days after they were received from the dealer, for the purpose of determining whether the calcium and inorganic phosphorus in the blood of rabbits, as they come to the laboratory from outside sources, differed in any way from that of rabbits living under the usual laboratory conditions. The results of this investigation are presented in the present paper in the form of a comparison of group means, variability and distribution frequencies.

Material and Methods.

The results to be reported are based on determinations of calcium and inorganic phosphorus in one sampe of blood from each of 111 male rabbits distributed as follows:

Oct. 8, 1926	10	rabbits
Nov. 17, 1926	10	"
Jan. 7–14, 1927	21	"
Feb. 4, 1927	11	"
Mar. 11, 1927	32	"
Apr. 29, 1927	10	"
May 25, 1927	9	"
June 8, 1927	8	"

The blood of the first group of animals and of some of those in the third group was not examined until they had been in the laboratory about 10 days; in other cases, the analyses were made between 1 and 3 days after the animals were received from the dealer.

These animals were comparable with those used for repeated determinations of calcium and inorganic phosphorus. In fact, the observations on the October group of this series were continued, constituting Group I of the series of repeated determinations already reported upon, while parts of the January and March animals formed Groups III and IV respectively of the first series.

Calcium and inorganic phosphorus were determined in accordance with the methods described in the previous paper (1).

The results of these determinations have been combined and analyzed as a single group in order to provide a basis for direct comparison with results obtained by repeated examination. For convenience, the series of repeated determinations will be designated as Series I, and the single determinations as Series II. A summary of results is given in Table I which, in addition to the values for Series II, contains the highest and lowest group values for Series I as well as the combined values for all groups of the series. As an aid to the interpretation of these results, the differences between mean values and the relation of these differences to their probable errors have also been tabulated (Table II). Tables III to V give the distribution frequencies for Series II with corresponding combined values for Series I. These relations are shown graphically in Text-figs. 1 to 7.

RESULTS.

The results of the investigation are summarized in Tables I to V and Text-figs. 1 to 7.

TABLE I.

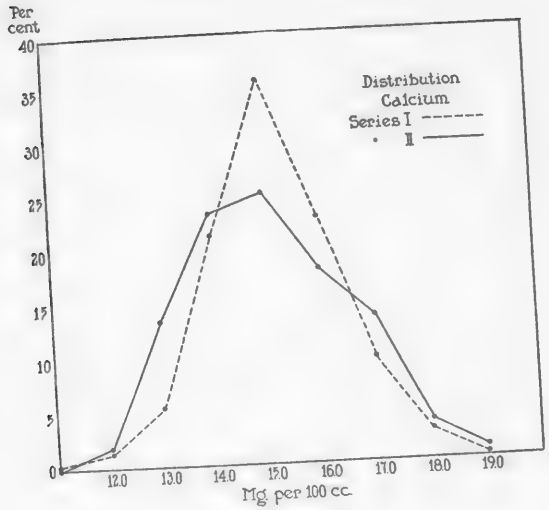
Values for Series of Single and Repeated Determinations of Calcium and Inorganic Phosphorus.

	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	*mg. per 100 cc.*	*per cent*
Calcium			
Series I High	15.8±0.06	1.12	7.09
Low	15.3±0.06	1.30	8.50
Combined	15.6±0.03	1.25	8.01
Series II	15.4±0.09	1.42	9.20
Inorganic phosphorus			
Series I High	4.96±0.04	0.80	16.13
Low	4.11±0.04	0.51	12.41
Combined	4.51±0.02	0.78	17.29
Series II	5.40±0.06	0.91	16.85
	Absolute values	Absolute values	
Sum			
Series I High	20.8±0.06	1.01	4.86
Low	19.6±0.06	1.31	6.68
Combined	20.1±0.04	1.29	6.42
Series II	20.8±0.11	1.70	8.17
Product			
Series I High	78.1±0.6	10.8	13.83
Low	64.0±0.8	10.6	16.56
Combined	70.2±0.3	12.0	17.09
Series II	83.3±1.03	16.0	19.22
Calcium-phosphorus ratio			
Series I High	3.83±0.04	0.53	13.84
Low	3.30±0.04	0.65	19.70
Combined	3.57±0.02	0.72	20.17
Series II	2.95±0.04	0.57	19.32
Product-sum ratio			
Series I High	3.75±0.02	0.42	11.20
Low	3.24±0.02	0.33	10.19
Combined	3.47±0.01	0.44	12.68
Series II	3.95±0.03	0.51	12.91
Sum of two ratios			
Series I High	7.07±0.03	0.41	5.80
Low	7.02±0.02	0.45	6.41
Combined	7.04±0.01	0.40	5.68
Series II	6.91±0.02	0.37	5.35

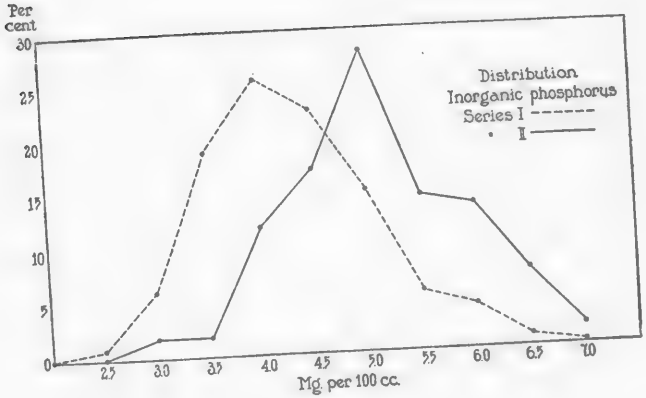
TABLE II.

Differences between Group Means as Compared with Their Probable Errors.

	Difference	$\frac{\text{Difference}}{P.E.}$
Calcium		
1. Series I, highest and lowest values	0.5 mg.	6
2. Series I, combined value and Series II	0.2 "	2
3. Series I, combined and highest values	0.2 "	3
4. Series I, combined and lowest values	0.3 "	4
Phosphorus		
1. Series I, highest and lowest values	0.85 mg.	15
2. Series I, combined value and Series II	0.89 "	14
3. Series I, combined and highest values	0.45 "	10
4. Series I, combined and lowest values	0.40 "	9
Sum		
1. Series I, highest and lowest values	1.2	14
2. Series I, combined value and Series II	0.7	6
3. Series I, combined and highest values	0.7	10
4. Series I, combined and lowest values	0.5	7
Product		
1. Series I, highest and lowest values	14.1	14
2. Series I, combined value and Series II	13.1	9
3. Series I, combined and highest values	7.9	12
4. Series I, combined and lowest values	6.2	7
Ratio of calcium to phosphorus		
1. Series I, highest and lowest values	0.53	9
2. Series I, combined value and Series II	0.62	15
3. Series I, combined and highest values	0.26	6
4. Series I, combined and lowest values	0.27	6
Ratio of product to sum		
1. Series I, highest and lowest values	0.51	18
2. Series I, combined value and Series II	0.48	15
3. Series I, combined and highest values	0.28	13
4. Series I, combined and lowest values	0.23	11
Sum of two ratios		
1. Series I, highest and lowest values	0.05	1
2. Series I, combined value and Series II	0.13	6
3. Series I, combined and highest values	0.03	Less than *P. E.*
4. Series I, combined and lowest values	0.02	Less than *P. E.*



TEXT-FIG. 1.

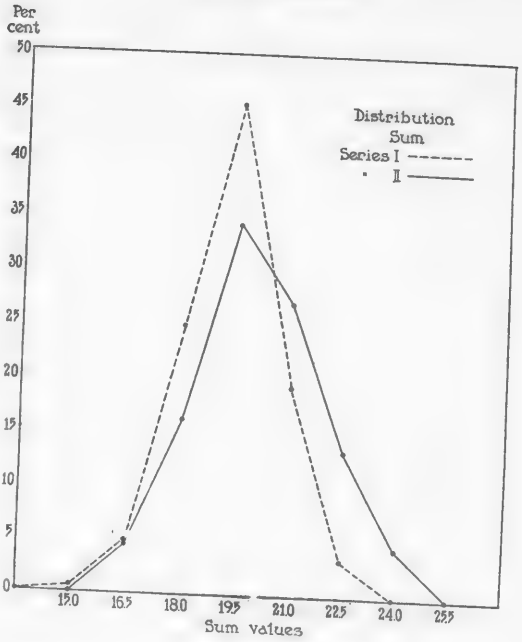


TEXT-FIG. 2.

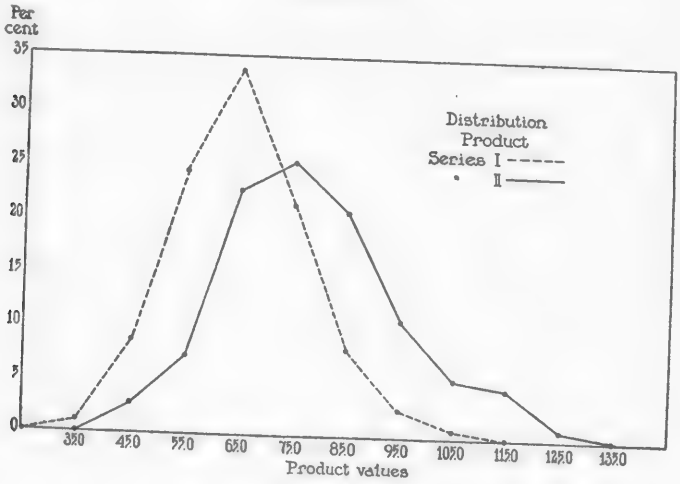
TABLE III.

Distribution of Values for Calcium and Inorganic Phosphorus.

Calcium	Series I	Series II	Inorganic phosphorus	Series I	Series II
mg. per 100 cc.	*per cent*	*per cent*	*mg. per 100 cc.*	*per cent*	*per cent*
11.0	0.34		2.00		
12.0	1.33	1.80	2.50	0.85	
13.0	5.66	13.52	3.00	6.18	1.80
14.0	21.32	23.42	3.50	18.89	1.80
15.0	35.77	25.23	4.00	25.60	11.92
16.0	22.79	18.02	4.50	22.58	17.12
17.0	9.67	13.52	5.00	15.05	27.92
18.0	2.66	3.60	5.50	5.52	14.41
19.0	0.17	0.90	6.00	4.02	13.52
20.0			6.50	0.99	7.20
21.0	0.17		7.00	0.17	1.80
22.0	0.17		7.50	0.17	1.80
			8.00		0.90



TEXT-FIG. 3.

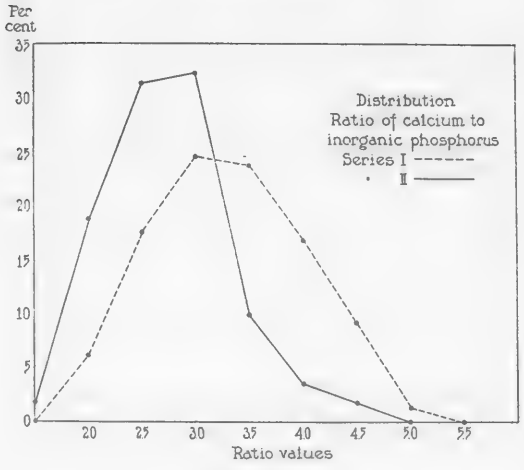


TEXT-FIG. 4.

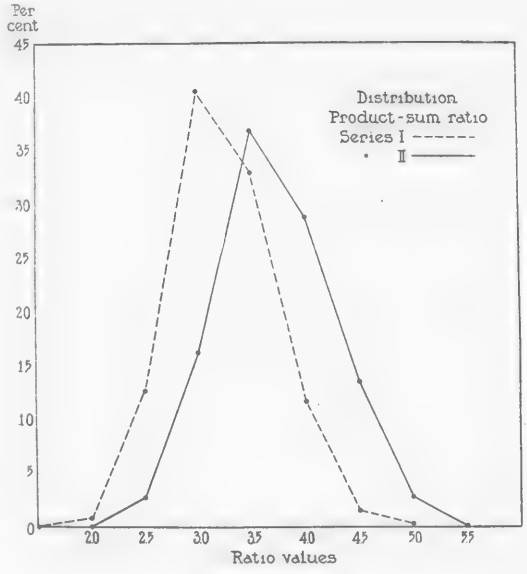
TABLE IV.

Distribution of Values for Sum and Product.

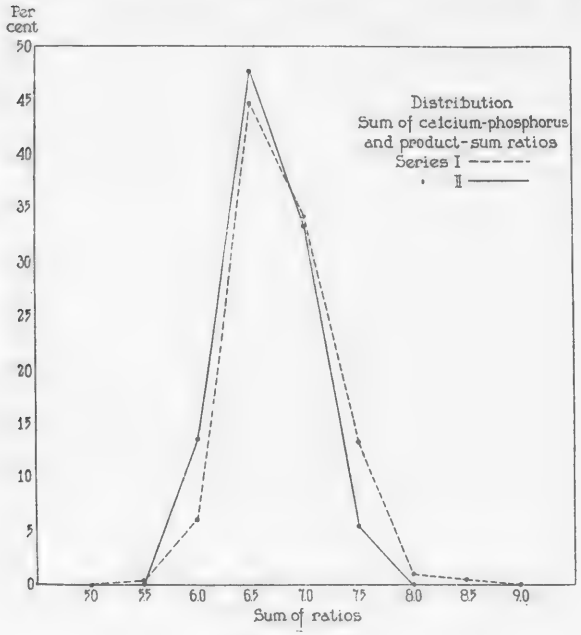
Sum values	Series I	Series II	Product values	Series I	Series II
	per cent	*per cent*		*per cent*	*per cent*
15.0	0.51		35.0	1.00	
16.5	4.86	4.50	45.0	8.53	2.70
18.0	24.79	16.22	55.0	24.30	7.20
19.5	45.39	34.23	65.0	33.67	22.52
21.0	19.44	27.03	75.0	21.28	25.22
22.5	3.35	13.52	85.0	8.04	20.72
24.0		3.60	95.0	2.51	10.81
25.5	0.17	0.90	105.0	0.66	5.40
27.0	0.17		115.0		4.50
			125.0		0.90



TEXT-FIG. 5.



TEXT-FIG. 6.



TEXT-FIG. 7.

TABLE V.

Distribution of Values for Ratios of Calcium to Inorganic Phosphorus and Product to Sum and for the Sum of the Two Ratios.

Calcium-phosphorus ratio values	Series I	Series II	Product-sum ratio values	Series I	Series II	Values for sum of two ratios	Series I	Series II
	per cent	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*
1.50		1.80	1.50			5.50	0.34	
2.00	6.20	18.92	2.00	0.84		6.00	6.03	13.51
2.50	17.59	31.53	2.50	12.73	1.80	6.50	44.73	47.75
3.00	24.63	32.43	3.00	40.54	16.21	7.00	34.17	33.34
3.50	23.79	9.91	3.50	32.49	36.93	7.50	13.22	5.40
4.00	16.76	3.60	4.00	11.72	28.83	8.00	1.01	
4.50	9.21	1.80	4.50	1.51	13.52	8.50	0.51	
5.00	1.34		5.00	0.17	2.70			
5.50	0.17							
6.00	0.34							

DISCUSSION AND CONCLUSIONS.

From an analysis of the results obtained by repeated determinations of calcium and inorganic phosphorus in the blood of 4 groups of rabbits, it was found that, under the conditions employed, both the absolute and relative amounts of these substances were subject to considerable variation. The question to be determined by the present investigation is whether a change in the experimental conditions which eliminates the factors of increasing age and prolonged cage life in the laboratory tends to produce results of the same or of a different order.

The mean value for calcium (Table I) is well within the limits established for animals living under laboratory conditions, and the value for the sum of the calcium and inorganic phosphorus is the same as the highest mean value for the previous series, but in no other case does the mean for the present group of animals fall within these limits. The mean value for inorganic phosphorus is considerably higher; the product and the ratio of the product to the sum are also higher, while the ratio of calcium to phosphorus and the sum of the two ratios are lower.

In the case of calcium, the difference between the mean and the mean of the combined values of Series I is 0.2 mg. This difference is of the same order as that shown by the highest and lowest mean values of Series I as compared with the combined value, but is less than the extreme difference of 0.5 mg. One may conclude, therefore, that the mean value obtained for calcium, in the present instance, does not differ significantly from values obtained under the other experimental conditions (Tables I and II).

The difference between the mean value for inorganic phosphorus and the basic value for Series I is, however, greater than that between the highest and lowest mean values of the series, and is approximately twice as large as the difference between these values and the combined value for the series (Tables I and II). The value for inorganic phosphorus is definitely outside of the limits established for Series I, and the difference between the two sets of results is many times the probable error; so that, in this case, it is certain that the higher value for inorganic phosphorus is significant. In this connection, it is of interest to note that the highest value of Series I was given by the

youngest animals, and that the observation period on these animals, or the period of confinement in the laboratory, was the shortest.

A similar comparison of the figures given for the sum of calcium and inorganic phosphorus shows that the value obtained represents a deviation from our basic value which is comparable to that found for animals living under laboratory conditions. The mean value is in fact identical with that for the youngest group of animals so living, but the results differ in other respects as will be pointed out later.

The increase of the product value above the standard mean (Series I) is approximately equal to the extreme difference between group means of Series I and nearly twice the deviation shown by high and low values of the series from the standard mean. The shift in the product values assumes the same direction as that for the inorganic phosphorus content of the blood and has virtually the same significance.

The deviation of the calcium-phosphorus ratio from the combined value of Series I is greater than that shown by any of the groups in that series, and represents a definite downward shift. The ratio of the product to the sum changes nearly, but not quite as much in the opposite direction, giving a value for the sum of the two ratios which is again well below the limits for Series I.

The probability of the chance occurrence of differences between mean values of the magnitude of those obtained is indicated in Table II.

A comparison of the variability and of the distribution of the two sets of calcium and phosphorus determinations is just as instructive as a comparison of mean values, but no attempt will be made to follow this out in detail. By reference to Table I, it will be seen that, in most cases, the standard deviation for Series II exceeds the maximum value for Series I, while the coefficients of variation show a closer agreement and are in most cases of a comparable order.

The distribution frequencies are especially interesting in that they show differences in the composition of the two sets of results that are not brought out in any other way (Tables III to V and Text-figs. 1 to 7). For example, while the mean values for calcium show a comparatively close agreement, the composition of these means is quite different (Text-fig. 1). The distribution of individual determinations

covers essentially the same range, and both curves are fairly symmetrical. But, in Series I, a higher proportion of values is grouped about the mean, while in Series II the percentage of values in both the upper and lower ranges is greater, giving a larger standard deviation and a larger coefficient of variation.

In all other cases, irrespective of the agreement between means or of differences in variability, the range covered by the observations on Series II differs from that of Series I. The form of the curves is much alike, indicating that they are produced by variants of the same class, but the axis of distribution moves upward or downward on the scale of values, showing that there are quantitative differences in the composition of the two sets of results. The curves for the sum of the ratios of calcium to inorganic phosphorus and of the product to the sum show the closest agreement, but the agreement is not as close as that shown by the 4 groups of Series I. It appears, therefore, that even in this case there is a significant difference, and this supposition is borne out by the fact that the difference between mean values is 6 times its probable error.

Comparison of the two sets of results leads to the conclusion that the calcium and inorganic phosphorus in the blood of rabbits, as they are brought into the laboratory, may differ to a considerable extent from that of rabbits living under laboratory conditions. Grant and Gates (2) found that caging for periods as long as 7 weeks increased the calcium content of the blood, but in the present instance inorganic phosphorus and the relations between calcium and phosphorus were affected more than the absolute values for calcium. The evidence presented in this paper is not sufficient to warrant any conclusion concerning the extent to which age as distinguished from living conditions may have affected the results. The influence of these and other factors is shown to better advantage by comparing the results of consecutive observations on the two series of animals. This aspect of the investigation will be presented in a subsequent paper.

SUMMARY.

Determinations of calcium and inorganic phosphorus were made on the blood of a large series of rabbits as they were received from the

dealer for the purpose of determining whether results obtained under these conditions differed from those obtained by repeated observations on animals living in the laboratory.

It was found that the results differed in the two cases, due largely to a decided difference in the inorganic phosphorus content of the blood; but the values for calcium were also affected.

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THE INTERCONVERTIBILITY OF "R" AND "S" FORMS OF PNEUMOCOCCUS.

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The terms "R" ("rough") and "S" ("smooth"), to designate two "variants" of the same bacterial species, were first employed by Arkwright (1). His observations were made on members of the colon-typhoid-dysentery group but were soon extended to many other organisms. Griffith (2) was the first to recognize two corresponding "variants" of Pneumococcus, and his findings were confirmed and extended by Reimann (3) and Amoss (4). The distinguishing features of the two forms of Pneumococcus may be summarized as follows: "S" forms are virulent; they produce the specific soluble substance, upon which type specificity depends (5); and they form colonies which have a smooth surface when examined by reflected light. "R" forms are avirulent; they do not produce the specific soluble substance; and they form colonies which have a rough surface when similarly examined.

Since the recognition of these two forms, much interest has centered in the question of their interchangeability. It is obvious that this subject, as Hadley (6) points out, has considerable significance, not only in the problem of epidemiology, but also in the interpretation of bacterial mutation.

It is proposed to review here the question of the interconvertibility of the two forms only in so far as it applies to Pneumococcus.

As early as 1891, Roger (7) reported that Pneumococcus, when grown in the sera of inoculated animals, became attenuated in virulence. Issaeff (8), in 1893, however, showed that this apparent loss of virulence was due to the protective action of the serum present, since bacteria, freed from the serum in which they had been grown, showed no alteration in virulence. Neufeld (9), in 1902, stated

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that a virulent strain of Pneumococcus, in the course of cultivation, may lose its virulence and its specific agglutinability. Friel (10), in 1915, found that pneumococci, when grown in homologous immune serum, became agglutinable and phagocytable in normal rabbit serum, and less virulent for mice than the untreated strains. Stryker (11), independently, about the same time, reported that the growth of virulent pneumococci in homologous immune serum produced (*a*) variations in agglutinability, (*b*) decrease in virulence, (*c*) inhibition of capsule formation, (*d*) increased phagocytability in normal serum, and (*e*) a change in antigenic properties. She found that the modified type was permanent while the culture was kept on plain media; but that reversion to the original type occurred following animal passage. These investigations preceded the recognition of "R" and "S" forms but the indications are that the avirulent organisms were of the "R" variety. Griffith (2), in the paper in which he first pointed out the morphological distinction between colonies of virulent and attenuated pneumococci, stated that, "an 'R' strain may revert in all respects to the 'S' type or may remain unchanged after many generations in subculture in plain blood broth." He was of the opinion that the constancy of the "R" variant was, in some degree at least, dependent upon the strength of the immune serum employed in its production. None of these investigators made use of single cell cultures, and the contrary results subsequently obtained by Amoss (4) and Reimann (12), were, in part, attributed by them to the fact that they employed pure line strains derived from single cell cultures. Amoss reported that the avirulent strains showed no tendency to revert to the parent type, and did not become virulent on repeated passage through mice. Reimann was unable to restore virulence or type specificity to a single cell "R" strain even after 105 mouse passages. Both Reimann and Amoss employed "R" cultures derived from Type I pneumococcus. Felton and Dougherty (13), by means of an automatic transferring device in a milk-containing medium, were able to restore virulence to a single cell culture of Type I pneumococcus which had become completely avirulent. Although no mention was made of "R" and "S" forms in their communication, it may be assumed that the avirulent cultures were of the "R" variety. They reported, however, that some cultures showed a maximal increase in virulence while others remained avirulent. Levinthal (14) also reported that he was able to effect reversion from "R" to "S" in single cell Type I cultures by growth in serum broth at 25°C., with subsequent passage of the cultures through mice.

The present study concerns itself, generally, with the question of the interconvertibility of "R" and "S" forms of Pneumococcus, and, more particularly, with the question of reversion from "R" to "S." In a previous communication (15), it was pointed out that virulence and type specificity could be restored to "R" forms by growth in anti-"R" sera. Interesting in this connection is the observation of Soule

(16). Investigating microbic dissociation in *B. subtilis*, he stated that, "by the incorporation of 'S' or 'R' immune sera in fluid media, 'R' forms may be obtained from 'S' forms, and 'S' forms from 'R' forms respectively."

Methods.

Both mass and single cell cultures of Types I, II, and III pneumococcus were employed in all experiments. The single cell strains were isolated according to the method of Avery and Leland (17). In this method the position of single organisms is first accurately determined on a thin film of agar spread on a specially designed cover-slip. The single organisms are then allowed to develop into colonies which are subcultured as desired.

The structure and surface appearance of colonies were studied on blood agar plates by means of a Zeiss binocular microscope specially adapted for the study of colony morphology. It is necessary to point out that colony appearance is only a relative guide to the nature of the organisms constituting a colony, and can never be absolutely relied upon to distinguish "R" and "S" forms. This is particularly true in the case of Type II pneumococcus. In this study, colony morphology alone was never considered as a final criterion, but was always confirmed by specific agglutination and virulence tests. The appearance of colonies also shows great variation with age, so that, except in certain instances, plate cultures 18–24 hours old were always selected for examination.

The immune sera employed were either the type-specific, diagnostic sera prepared from horses by the New York State Department of Health,[1] or sera obtained from animals immunized according to the method of Cole and Moore (18).

Unless otherwise indicated, subcultures were made twice in 24 hours, and incubated at 37°C.

EXPERIMENTAL.

I. Reversion from "R" to "S" in Mass Cultures.

A. By Animal Passage.

Mass cultures of "R" forms derived, respectively, from pneumococci of Types I, II, and III, were employed. In each instance these were stock laboratory "R" strains which had been maintained in artificial cultivation at least 2 years, and which showed no tendency to revert to the "S" form by growth in any of the usual media. These

[1] We are indebted to Dr. Augustus B. Wadsworth of the New York State Department of Health for the antipneumococcus horse sera employed in this investigation.

cultures were avirulent, 0.5 cc. of a young broth culture uniformly failing to kill white mice; they did not form capsules or produce the specific soluble substances; and formed only typical rough colonies on blood agar plates.

(*a*) *"R" Forms Derived from Type I* (*Strain I/192/R*).—It was not found possible to effect reversion of this strain by animal passage. This confirmed the results of Reimann, who had previously subjected the same strain to 105 consecutive mouse passages.

(*b*) *"R" Forms Derived from Type II* (*Strain D/39/R*).—3 cc. of a 6 hour growth in plain broth were injected into the peritoneum of a mouse and the animal killed in 4 hours. 1 cc. of the peritoneal washings was injected into a second mouse, and the process repeated after 6 hours, the third animal succumbing on the following day. Three further passages were made and a blood broth culture from the sixth mouse proved highly virulent, 0.000001 cc. causing death of an injected animal. The culture now showed only typical "S" colonies, gave a typical Type II agglutination, and produced an abundance of the specific soluble substance. Reversion to the "S" forms had been effected in all respects.

(*c*) *"R" Forms Derived from Type III* (*Strain M/3/R*).—Much greater difficulty was experienced in causing this strain to revert and it was only after twenty-eight successive mouse passages that it was accomplished. However, reversion did ultimately occur, and it was accompanied by the acquisition of all the characteristics of the "S" form, including maximal virulence.

B. By Growth in Anti-"R" Sera.

The same three "R" strains, derived from Types I, II, and III, respectively, were grown in plain broth, to which 10 per cent of anti-"R" serum obtained from a rabbit immunized to an "R" form was added. In the first experiments the serum employed was from an animal immunized to an "R" form derived from Type III (Strain M/3/R), but it was subsequently found that any anti-"R" serum, provided it was of sufficiently high titer, was equally effective. This phase of the problem will be discussed later.

(*a*) *"R" Forms Derived from Type I* (*Strain I/192/R*).—No change was effected in this culture even after 100 transfers in 10 per cent

anti-"R" serum. The surface of the colonies became somewhat less rough but there was no increase in virulence and no evidence of return to type specificity.

(*b*) *"R" Forms Derived from Type II (Strain D/39/R).*—This strain, which had remained unchanged after many transfers in blood broth, but which could be reverted to the "S" type by animal passage, showed an unexpected transformation when grown in anti-"R" serum. During four transfers there was no change in the character of the growth, the organisms settling to the bottom of the tube in agglutinated masses, as is usual with "R" forms when grown in anti-"R" sera. On the fifth transfer, however, the media became slightly turbid, and more definitely so on the sixth subculture. On the seventh transfer, the culture showed a uniform turbidity, gave a specific Type II reaction, and proved highly virulent for white mice, 0.000001 cc. causing death. Examination of the colonies on plates showed only typical "S" forms. Evidently complete reversion had been effected.

(*c*) *"R" Forms Derived from Type III (Strain M/3/R).*—"R" forms of Type III pneumococcus reverted to the "S" type, when grown in anti-"R" sera, in a manner similar to "R" forms of Type II. After a variable number of transfers, usually between eight and twelve, the growth became diffuse, smooth colonies appeared on plates, and type specificity, accompanied by maximal virulence, was restored. It is of interest to note that, once the process was initiated, it was only a matter of two or three further transfers before the entire culture had assumed the "S" form, and "R" colonies could not be found.

From these experiments the conclusion may be drawn that, in certain instances at least, mass cultures of avirulent, "R" forms of pneumococci possess the ability to revert to virulent, type-specific, "S" forms, and that the change can be effected by *in vitro* as well as by *in vivo* methods.

Two questions then presented themselves: first, is reversion due to the presence of certain individual, undetected "S" forms within the mass "R" cultures, or do all "R" cells individually possess the ability to revert; and secondly, can reversion be effected by growth in an artificial medium other than that containing anti-"R" serum?

II. Reversion from "R" to "S" in Single Cell Cultures.

Four single cell strains were obtained from each of the three "R" cultures originally derived from the three specific types of Pneumococcus, and their characteristics definitely determined. They were avirulent, 0.5 cc. of broth culture producing no effect on injection into white mice; they showed no evidence of type specificity, and formed only rough colonies on plates. Reversion was then attempted both by animal passage and by growth in anti-"R" sera.

A. By Animal Passage.

The results obtained when single cell cultures were employed so closely parallel those outlined in the case of mass cultures that it is not necessary to give the data in detail. In no instance was it possible by animal passage to effect reversion with the single cell "R" forms derived from Type I (Strain I/192/R). The single cell "R" cultures derived from Types II and III (Strains D/39/R and M/3/R) reverted to the "S" form after practically the same number of mouse passages as was necessary when mass cultures were used. Little or no individual differences were found to exist in the various single cell cultures derived from the same "R" strain.

B. By Growth in Anti-"R" Sera.

The same dilution of serum was used which has been effective in producing reversion in mass cultures. Again entirely similar results were obtained, and practically no individual difference was found to exist in the various single cell strains isolated from the same mass culture. The "R" forms of Type I (Strain I/192/R) uniformly failed to revert; all the "R" forms of Type II (Strain D/39/R) reverted in from five to ten transfers; and the "R" forms of Type III (Strain M/3/R) in from ten to twenty transfers. The "R" forms of Type I were again carried in subculture up to 100 transfers in media containing anti-"R" serum without success.

From the results with single cell cultures, it seems fair to conclude that the reversion of an "R" strain to the "S" type does not depend upon the presence of an admixture of both forms within the culture, but rather it seems not unlikely that each individual "R" organism either may, or may not, possess the ability to revert to the "S" type.

Results with "R" Strains Derived from Other Type I Cultures.

The "R" strain of Type I which was used in the preceding experiments was a stock strain which had been under artificial cultivation for a period of many years. In order to determine whether this inability to revert was a property of all "R" organisms derived from Type I, or only of this particular strain, other "R" cultures derived from Type I were employed. These "R" forms were obtained by growing two Type I "S" strains, recently isolated from cases of lobar pneumonia, in 25 per cent Type I serum. After eight transfers in this medium, four typical "R" colonies, two from each strain, were selected, and reversion was attempted both in mass cultures grown from the single colonies, and in single cell cultures derived from the same colonies. That these cultures were definitely of the "R" forms was repeatedly confirmed by colony appearance, lack of type-specific agglutination, and loss of virulence. In this instance, uniform results were not obtained; but it is of interest to record that the single cell cultures behaved in precisely the same manner as the whole colony cultures from which the single cells were derived. Of the four colony cultures, it was possible, by the method of animal passage, to restore type specificity and virulence to only one. Likewise it was possible to effect reversion by animal passage in single cell cultures derived from this colony and not in the case of the others.

Reversion of these "R" strains of Type I was then attempted in anti-"R" sera. Success was attained with the culture whose virulence could be restored by animal passage but not with the others. Forty transfers in anti-"R" serum broth were necessary and both single cell and mass cultures reverted. Here, again, reversion was accompanied by the acquisition of all the characteristics of the "S" type, including maximal virulence. Thus it appears, at least in so far as Type I is concerned, that there are varying degrees of constancy of the "R" variant. Whether this constancy depends upon the strength of the immune serum employed in the production of the "R" forms as Griffith (2) suggests, or upon the number of transfers in immune serum to which the "R" variant has been exposed, further work is necessary to determine. So far, however, it has always been found possible to effect reversion of "R" forms of Types II and III, single cell as well as mass cultures, both by animal passage and by growth in anti-"R" sera.

Conversion of "S" to "R".

It was thought that possibly some light could be thrown on the difficulty experienced in causing "R" forms of Type I to revert to the "S" type by studying the reverse process ("S" → "R") in greater detail than had previously been done. It was hoped that this procedure would also be of assistance in the interpretation of certain puzzling forms of colonies which were occasionally encountered when "R" forms were grown in anti-"R" sera. "S" strains of Types I, II, and III, were grown through repeated transfers in broth containing the homologous antisera in dilutions varying from 1:1, 1:2, etc., to 1:256.

Type I "S" (Strains I/192/2, "P," and "G").—Each strain went through a more or less similar transformation of which the following account of Strain I/192/2 is typical. Unless otherwise indicated, the description of colonies applies to plate cultures 18–24 hours old.

The growth in 100 per cent Type I serum was poor and plates showed only typical "S" colonies for three transfers. In all serum dilutions, where growth was more abundant, the colonies also remained typically "S" for three transfers before a change was produced. After the fourth transfer, a change became apparent in all dilutions from 1:2 to 1:64—there was great variation in the size of the colonies, without any change in their surface appearance or outline. In higher dilutions only typically "S" colonies occurred. On the fifth transfer, in dilutions 1:2 to 1:64, a small, flat, indistinct, rough colony made its appearance in frequent numbers. As these plates aged (48 hours) a most bizarre picture was produced. Many of the large, smooth colonies developed nibbled areas at the periphery, and the small, flat, rough ones produced a great variety of forms—some developed smooth, nodular papillæ; some a mulberry-like appearance; while others appeared to be undergoing autolysis. After the sixth transfer, the plate presented a still more unusual appearance. Many of the large, smooth colonies showed pronounced nibbling and irregularity of outline, while all the colonies showed a complete lack of uniformity in both size and surface appearance. Occasional "rough" colonies were observed. On the seventh transfer, the majority of the colonies possessed a definitely "rough" surface, but they showed a peculiar "watery" appearance which distinguished them from typical "R" forms. After the eighth transfer, typically "rough" colonies could be recognized but even up

to the fourteenth transfer the small, irregular, rough colony still persisted. Repeated attempts were made to isolate these small, irregular colonies and to establish stable, intermediate forms. These efforts were not successful. Subcultures showed mixtures of the atypical intermediate forms and true "S" colonies.

Type II "S" (Strain D/39/36/2).—In the case of Type II it becomes much more difficult to describe the colony appearance, for the distinction between "R" and "S" forms was much less pronounced and had a purely relative value. In fact it was not unusual to find that a virulent Type II culture would give rise to colonies which appeared rough; while, on the other hand, colonies which were apparently smooth, especially when grown in blood or serum broth, were not necessarily type-specific. However, it is possible to say that the transformation from Type II "S" to the "R" form occurred much more readily than in Type I, one exposure to type-specific serum often proving sufficient. Moreover, the unusual transition picture described in Type I was not encountered, possibly because of the readiness with which the transition to the "R" form occurred.

Type III "S" (Strain A66/47/2).—With Type III it was always possible to distinguish "R" and "S" colonies. After one transfer in homologous anti-"S" serum, in all dilutions, only "S" colonies occurred. After two transfers only a few "S" colonies persisted in serum dilutions 1:2 and 1:4; in higher dilutions only "S" forms were apparent. After four transfers, dilutions 1:2 and 1:4 showed only "R" forms; in dilution 1:8 only two "S" colonies remained. After the sixth transfer, all the colonies were of the "R" type in dilutions 1:2, 1:4, and 1:8, while in higher dilutions, only "S" forms persisted. No intermediate forms were observed, all colonies being definitely either "R" or "S."

From these experiments it is possible to state that Type I "S" pneumococcus is by far the most difficult to convert to the "R" form, requiring ten to fifteen transfers in serum dilutions of 1:2 and 1:4. Even after this number of transfers "intermediate" colonies exist which readily revert to the "S" type. On the other hand, Types II "S" and III "S" are easily converted to the "R" form, and in each instance the change is much more abrupt and complete than in the case of Type I. This fact may offer a partial explanation of the difficulty experienced in the reverse process of transforming "R" forms derived from Type I to the "S" type.

The Rôle of Anti-"R" Serum in Reversion by in Vitro Methods.

Having established the fact that, in the majority of instances, it is possible to transform "R" cultures of Pneumococcus to the "S" type by growth in anti-"R" sera, attempts were made to effect the change in media which did not contain anti-"R" antibodies. Experiments were also carried out to determine the titer and concentraton of serum best suited for the purpose.

Sera were obtained from a series of normal individuals and animals, and also from animals which had been immunized to either the "R" or "S" forms of Pneumococcus. Attention is drawn to the observation of Avery and Heidelberger (19) that type-specific, antipneumococcus

TABLE I.

Reversion "R" to "S"—Type II Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type II (D/39/R). Single cell culture	Normal chicken	Nil	100	No change
	" rabbit	1:5	100	" "
	" sheep	1:20	100	" "
	" horse	1:20	100	" "
	" guinea pig	1:320	30	Partial reversion
	" human	1:640	8	Complete "
	Immune rabbit	1:3200	5	" "
	" horse	1:3200	5	" "
	Plain broth		100	No change

sera contain not only dominant, type-specific (anti-"S") antibodies, but also antibodies reacting with the protein substance, which is common to all pneumococci. Reimann (12) subsequently showed that sera prepared with "R" forms are immunologically similar to sera prepared with the protein of Pneumococcus. In consequence it was possible to use not only anti-"R" sera prepared against any "R" strain regardless of its type derivation, but also anti-"S" sera of the three specific types, all of which possess, in common, anti-"R" antibodies. The anti-"R" titer of the sera was determined by the so called thread reaction, and single cell cultures were employed.

The results obtained with "R" forms of Type II pneumococcus are set forth in Table I. It is seen that complete reversion occurred only in those sera of which the anti-"R" titer was above 1:320. In normal guinea pig serum, the titer of which was 1:320, a partial reversion was observed. Under these conditions the organisms lost their ability to thread; the colonies became smoother; and the virulence of the culture was increased, but not to a maximal degree. It was not probable in this instance that the growth was made up of a mixture of "R" and "S" forms; for all the colonies were of similar appearance and sub-

TABLE II.

Reversion "R" to "S"—Type III Pneumococcus. Growth in Normal and Anti-"R" Sera.

Culture	Serum broth dilutions 10 per cent	Anti-"R" titer of sera	No. transfers	Result
"R" forms of Type III (M/3/R). Single cell culture	Normal chicken	Nil	50	No change
	" goat	"	50	" "
	" horse	1:10	50	" "
	" sheep	1:20	50	" "
	" rabbit	1:20	25	Complete reversion
	" guinea pig	1:80	50	Partial " ?
	Immune goat	1:200	50	No change
	Normal human	1:640	50	" "
	Immune sheep	1:800	50	" "
	" rabbit	1:3200	18	Complete reversion
	" horse	1:6400	12	" "
	Plain broth		50	No change

cultures made from them individually possessed the same characteristics. Even when subcultured for twenty further transfers in the same media complete reversion did not occur. Growth in serum broth with an anti-"R" titer of less than 1:320 induced little change. In some instances the organisms ceased to give a thread reaction, but there was slight, if any, increase in virulence. They did not give a type-specific agglutination and were still unquestionably "R" forms.

The corresponding results obtained with "R" forms of Type III are outlined in Table II. Reversion again occurred promptly in those sera the anti-"R" content of which was highest, and not at all in those

which did not contain anti-"R" antibodies. A partial exception occurred in the case of normal rabbit serum, the anti-"R" titer of which was only 1:20. Here complete reversion was effected after twenty-five transfers. This was the only instance encountered in which the transformation took place in sera of low anti-"R" titer. This observation suggests that, while reversion unquestionably occurs much more readily in sera of high anti-"R" content, it may take place occasionally even in sera of low anti-"R" titer.

The results obtained by growing "R" forms of Type III in normal guinea pig sera are of some interest. A great variety of colony appearance was produced which closely resembled that previously described

TABLE III.

Optimal Concentration of Anti-"R" Serum for Reversion.

Culture	Serum concentration	No. transfers to effect reversion
"R" forms of Type II (Strain D/39/R)	25 per cent	8
	10 " "	4
	5 " "	10
	1 " "	No change
"R" forms of Type III (Strain M/3/R)	25 " "	16
	10 " "	7
	5 " "	20
	1 " "	No change

in the degradation of Type I "S" cultures. After fifteen transfers in this medium, colonies quite irregular in size, outline, and surface appearance were observed. In addition to the more usual "rough" forms there were small, indistinct, flat, smooth colonies; small, rough ones from which projected smooth nodular papillæ; and medium sized, apparently smooth colonies. All of these types of colonies appeared on plate cultures after 18 hours incubation. After ten further transfers only smooth colonies occurred but they were not of the large, mucoid, typical, Type III variety and subcultures from them were avirulent and not type-specific. Continued transfers in normal guinea pig serum produced no change and when transferred back to plain broth only typical "R" forms resulted. Possibly this culture represented an unstable intermediate form.

Optimal Concentration of Anti-"R" Sera for Reversion.

Cultures of "R" forms of Types II and III pneumococcus were grown for successive transfers in broth containing varying dilutions of anti-"R" rabbit serum of known titer. The results are recorded in Table III. The anti-"R" titer of the serum used, as determined by the thread reaction, was 1:6400. In all experiments 10 per cent was found to be the optimal concentration. Reversion also occurred in serum dilutions of 25 per cent and 5 per cent but a greater number of transfers was necessary.

Experiments were also done with sera which had been inactivated by heating at 56° for ½ hour, and it was found that this procedure did not reduce the anti-"R" titer nor destroy its ability to cause reversion. No work was done under anaerobic conditions nor were the sera of animals immunized to the nucleoprotein of Pneumococcus tested.

DISCUSSION.

The present work establishes the fact, that, in the majority of instances, it is possible to cause both mass and single cell "R" cultures of Pneumococcus to revert to the "S" type. It was found that there are differences in the stability of the "R" forms in the three types, "R" forms of Type I being the most permanent. This corresponds to the greater resistance to degradation exhibited by Type I "S." Absolutely irreversible "R" organisms were encountered only in Type I. No attempt was made to determine whether it is possible to produce such irreversible forms in Types II and III.

It is possible to cause reversion from "R" to "S" pneumococci by an *in vitro* as well as an *in vivo* method. Reversion from "R" to "S," whether effected *in vitro* or *in vivo*, is always accompanied by the acquisition of maximal virulence. The *in vitro* method consists in growing "R" organisms in anti-"R" sera, the optimal concentration of which is 10 per cent. Growth in sera which do not contain anti-"R" antibodies, has, with one partial exception, not been found to be effective in producing the transformation. The partial exception was that of a normal rabbit serum, the anti-"R" titer of which, as determined by the thread reaction, was 1:20. "R" cultures have invariably reverted to the specific type of Pneumococcus from which the

"R" organisms were originally derived. After reversion, the culture possesses maximal virulence, elaborates the specific soluble substance, and is type-specific. No explanation is offered for the causes responsible for the change.

In our experience single cell strains have always reacted in the same manner as the mass cultures from which they were isolated. This finding argues against the hypothesis that the virulence of a culture depends upon the relative number of "R" and "S" forms of which it is composed.

The observation that normal human sera contain sufficient anti-"R" antibodies to cause reversion of some "R" cultures is thought to be of some interest.

It is not the purpose of this paper to enter into a discussion of the epidemiological significance of these findings, nor the bearing they may have on the subject of bacterial mutation.

CONCLUSIONS.

1. Single cell cultures of "R" pneumococci may revert in all respects to the "S" type.

2. In all instances in which reversion has occurred the "R" forms have invariably reverted to the same specific type from which they were originally derived.

3. The transformation "R" to "S" may be effected *in vitro* by growth in anti-"R" sera, as well as *in vivo* by the method of animal passage.

4. Reversion from "R" to "S," whether effected *in vivo* or *in vitro*, is always accompanied by the acquisition of maximal virulence.

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STUDIES UPON THE PHYSIOLOGICAL ACTION OF HEMATOPORPHYRIN.

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INTRODUCTION.

The behavior of animals having non-pigmented skin when injected with a suitable dose of hematoporphyrin, is well known. If such an animal is kept in the dark, no striking difference in behavior occurs from that observed in a normal, uninjected animal kept under the same conditions. On the other hand, if an animal having received such a dose of hematoporphyrin is exposed to sunlight or to strong, well cooled artificial light it soon becomes restless, violently scratches the nose, ears and other parts of the body, frequently jumps high into the air and shows livid discoloration of the skin; the respiration is rapid and the pulse rate increased. Following a short period of such activity the animal grows very weak and listless, gradually falls into coma, develops slow, very deep respiration, a slow pulse, and soon dies. This physiological state has been called hematoporphyrin shock.

The anatomical changes taking place in an animal subjected to this experiment are quite inadequate to explain the cause of death, or to account for the nature of the rôle which either light or hematoporphyrin plays in the process.

Soon after exposure to light is begun, edema of the skin appears, in white mice first becoming manifest in areas unprotected by hair, such as the eyelids, ears and tail. Rigor mortis occurs rapidly. The blood is very dark and clots slowly after death. Hemorrhages may be found in the internal organs, and in some cases blood is present in the lumen of the intestine. The mesenteric vessels, which after death are always found to be distended with blood, occasionally show marked infiltration of their walls with leucocytes, but this finding is frequently absent in even the most severe cases. Slight infiltration of leucocytes in the edematous skin may occur.

Animals injected with such a fatal, sensitizing dose of hematoporphyrin and then exposed to diffused daylight do not develop the acute symptoms of acute hematoporphyrin shock just described. They show signs of irritation, and after repeated injections with hematoporphyrin and exposures to light, their ears become necrotic and drop off. Following a period of cachexia, such animals die, but show no significant or consistent anatomical changes. Alterations in the central nervous system have been described by Eppinger and Arnstein (1).

Shock is never produced by exposure of only a small portion of the body to sunlight after injection of hematoporphyrin. The skin of the exposed area, however, becomes edematous and red, and after a few days shows necrosis. A deep ulcer develops at this point which heals slowly. Microscopically one finds thrombosis of the small vessels in the margin of the ulcer, with necrosis and cellular infiltration of its surface.

After intravenous, intraperitoneal or subcutaneous injection, hematoporphyrin may soon be found in the stomach and intestine, bile and urine. The blood serum at this time contains only a faint trace of hematoporphyrin.

We thus see that hematoporphyrin in suitable doses, *per se*, produces no marked effect when injected into animals. When, however, such animals are exposed to sunlight soon after injection of the drug, very characteristic and striking symptoms appear which rapidly progress to a state of coma soon ending in death.

Anatomical studies performed upon such animals reveal no adequate explanation of the mechanism by which the combined action of light and hematoporphyrin produces death. Comparatively little is known of the physiological state of the body produced by the combined action of hematoporphyrin and light. As the result of a series of experiments performed *in vitro*, Gaffron (2) has concluded that hematoporphyrin acts as a catalyzer which, under the influence of light, causes acceleration of oxidation and accordingly rapid consumption of oxygen.

The following experiments have been performed in the attempt to elucidate further the physiological characteristics of the condition which has been described as hematoporphyrin shock, as well as to test the validity of the theory concerning the nature of the action of hematoporphyrin proposed by Gaffron (2).

All of the hematoporphyrin used in these experiments was prepared by the well known method of Nencki (3).

Effect of Feeding Hematoporphyrin.

An attempt to sensitize white mice to light by feeding hematoporphyrin was ineffective.

Four mice were fed upon bread only, soaked in a weak solution of hematoporphyrin and dried. Two of these animals were kept constantly exposed to diffused daylight in the laboratory for 2 months, while the other two were kept in the dark. Upon numerous occasions during this time all four mice were exposed to direct bright sunlight for several hours. No changes in the skin, general appearance or behavior were noted in any of the four animals. Hematoporphyrin was constantly present in the stools but was never found in the urine.

The Relationship of the Cutaneous Pigment to the Effect of Hematoporphyrin and Light.

Since it is well known that exposure to light after injection of hematoporphyrin affects only white animals or those whose skin is slightly pigmented, it was of interest to determine whether this protection is of purely physical nature or in some other way associated with the pigment.

Albino and slightly pigmented rats and mice were stained a dark blue-black color with Verhoeff's hematoxylin and injected, along with unstained controls, with doses of hematoporphyrin adequate to cause death of the controls upon exposure to sunlight. A number of both groups of animals were exposed to sunlight and the others kept either in the dark or in the subdued light of the laboratory. The unstained controls promptly developed hematoporphyrin shock when exposed to sunlight, while the darkly stained animals, which had just received the same amounts of hematoporphyrin as had the controls, were completely protected from the sun's rays. Repeated exposure of these animals to sunlight produced no untoward effects.

It was clear from this experiment that the same degree of protection offered by cutaneous pigment can be produced by staining the skin of non-pigmented animals. Accordingly, one seems justified in assuming that the natural pigment of the skin plays only a physical rôle in the protection of animals injected with hematoporphyrin from light.

None of the stained or control animals kept in the dark or in subdued light showed any changes in their general behavior. Frozen sections of the skin of these two groups of animals, as well as those exposed to sunlight and normal animals, were made and treated with dioxyphenylalanine (Dopa reaction) to see if any alteration in the cutaneous pigment had been produced by any of these procedures (4). No changes of this nature could be demonstrated.

Sensitizing Effect of the Blood of Animals in Hematoporphyrin Shock.

It has so far been impossible to demonstrate any sensitizing substance in the blood of animals during the period of hematoporphyrin shock.

White mice were injected intraperitoneally with 1.5 cc., or intravenously with ½ cc. each, of the heart's blood of guinea pigs drawn at the height of hematoporphyrin shock and kept in diffused daylight. No difference could be seen between

the behavior and general appearance of these animals and mice similarly injected with an equal amount of normal guinea pig blood and kept under the same experimental conditions. All of these mice were very ill for a few days following the injection, but recovered.

Since injection of blood from another species was obviously undesirable, the sensitizing effect of the blood of animals in hematoporphyrin shock was tested in another way.

A parabiosis of two young white rats of the same litter and sex was performed. 6 weeks after the operation, when both rats were in good condition, one of these animals was injected intravenously with trypan blue. The skin of the non-injected rat, as well as that of the injected one, soon became colored by the dye, thus proving the existence of adequate vascular connections between the circulations of the two animals. The first rat was then injected subcutaneously with 4 cc. of a 1 per cent alkaline solution of hematoporphyrin and exposed to bright sunlight, while the second rat was protected from the rays of the sun by a wooden box which was cut out on one side so as not to obstruct the blood vessels connecting the circulations of the two animals. The injected and exposed rat soon showed the characteristic symptoms of sensitization to light, passed into hematoporphyrin shock and died. The other animal, protected from the light, showed no changes in appearance or behavior. Just before the death of the first rat, the two animals were separated and the wound in the side of the unexposed rat closed. This animal lived and appeared to be entirely normal during the several months it was kept in the laboratory. No pathological changes were found at autopsy.

Exposure of the Peritoneum to Light after Injection of Hematoporphyrin.

Two cats of about equal size were used for this experiment. One animal was given subcutaneously 15 cc. of 1 per cent alkaline hematoporphyrin and shortly afterwards, under ether anesthesia, the peritoneal cavities of both animals were opened widely, and exposed to bright sunlight. The peritoneum was kept moist with normal saline, and the rest of the body well protected from the light by towels. After 3 hours exposure of the peritoneal cavity to sunlight, the cat which had been injected with hematoporphyrin died. At this time the peritoneal cavity of the control animal, which was still in good condition, was closed and the anesthesia stopped. This latter animal quickly recovered.

This single experiment suggests that exposure of any other large surface of the body to sunlight after the injection of hematoporphyrin has the same effect as the exposure of non-pigmented skin. It was observed that the serosa of the intestines of the injected cat became very pale during the exposure to sunlight, while that of the normal animal showed marked hyperemia.

Effect upon the Blood Pressure of the Exposure to Strong Light of (a) Circulating Blood of Normal Animals, (b) Circulating Blood after Injection of Hematoporphyrin.

(a)

Reed (5), experimenting with normal dogs, reports a marked fall of blood pressure, sometimes reaching shock level, upon exposure to strong arclight of the blood alone. He interposed a glass tube, 3 mm. in diameter, between the divided

TABLE I.

(a) Exposure to Arclight and Sunlight of Circulating Blood of Normal Animals.

Type of experiment	Animal	Hours of exposure	Exposure	Mean blood pressure		Fall in blood pressure	Remarks
				Beginning of experiment	End of experiment		
			min.	*mm. Hg*	*mm. Hg*	*mm. Hg*	
Cannula interposed in femoral artery or connecting femoral artery and vein exposed to arclight	Dog 1	4.40 p.m.– 7.10 p.m.	150	115	80	35	
	Dog 2	4.16 p.m.– 5.30 p.m.	74	135	100	35	
	Dog 5	3.20 p.m.– 4.40 p.m.	80	140	110	30	
	Cat 101	3.15 p.m.– 5.00 p.m.	105	140	60	80	
Cannula connecting femoral artery and vein exposed to sunlight	Dog 8	11.16 a.m.–12.45 p.m.	89	130	130	0	Jan.
	Cat 21	11.16 a.m.– 1.15 p.m.	119	110	60	50	May
	Cat 24	10.52 a.m.–12.51 p.m.	119	110	60	50	May (spiral cannula)
	Cat 27	10.11 a.m.–11.40 a.m.	89	150	100	50	May (spiral cannula)

ends of the carotid artery, and exposed the blood flowing through this tube to a beam of light supplied by a powerful carbon arc. In some cases the tube was cleaned repeatedly during the experiment, while in others coagulation of the blood was prevented by injection of heparin. Reed remarks that the result was somewhat modified at times by the use of heparin.

In repeating Reed's experiments in slightly modified form upon cats and dogs such a great fall in blood pressure as he reports has not been observed.

In our experiments a quartz glass tube was either interposed between the cut ends of the divided femoral artery of the anesthetized animal, or it was used to connect the femoral artery and vein. Coagulation of the blood was prevented by injection of heparin. The blood of cats and dogs while flowing through such a tube was exposed, in some cases, to a cooled beam of light composed of the rays

TABLE II.

(*b*) *Exposure to Arclight and Sunlight of Circulating Blood after Injection of Hematoporphyrin.*

Type of experiment	Animal	Hours of exposure	Exposure	Mean blood pressure		Fall in blood pressure	Amount of hematoporphyrin injected
				Beginning of experiment	End of experiment		
			min.	*mm. Hg*	*mm. Hg*	*mm. Hg*	
Cannula femoral artery and vein. Intraven. inj. hematoporphyrin. Cannula exposed to arclight	Cat 20 (1,800 gm.)	4.00 p.m.–5.50 p.m.	110	135	30	105	Intraperiton. inj. 20 cc. 0.5% hematoporphyrin
	Cat 105 (2,100 gm.)	11.20 a.m.–1.30 p.m.	130	100	65	35	Intraven. inj. 15 cc. 0.8% hematoporphyrin
	Dog 2 (5,200 gm.)	5.30 p.m.–7.10 p.m.	100	100	90	10	Intraven. inj. 15 cc. 4% eosin
Cannula femoral artery and vein. Intraven. inj. hematoporphyrin. Cannula exposed to sunlight	Cat 22 (1,900 gm.)	11.30 a.m.–1.55 p.m.	145	120	80	40	May, 20 cc. 0.5% hematoporphyrin
	Cat 23 (1,850 gm.)	11.30 a.m.–2.00 p.m.	150	120	65	55	May, 20 cc. 0.5% hematoporphyrin (spiral cannula)

from a 1000 watt carbon arc, and in other instances to sunlight. The mean blood pressure was recorded directly from the carotid artery.

As seen in Table I, there occurred a considerable fall in mean blood pressure in all cases except Dog 8, in which no fall in pressure followed

89 minutes exposure of the circulating blood to direct sunlight. In no instance, however, did the mean blood pressure fall below 60 mm. Hg. In the experiments performed upon Cats 24 and 27, a spiral cannula, 3 mm. in diameter, was used as an exposure chamber for the blood instead of the simple glass tube employed in the other experiments. In this way, a much larger quantity of blood was constantly exposed.

From this group of experiments, therefore, it does not seem that exposure of the circulating blood alone to strong light produces any strikingly greater fall of arterial pressure than might be expected to take place following approximately 2 hours anesthesia carried out in the ordinary light of the laboratory.

(*b*)

In this group of animals, the preceding experiment was repeated immediately after the injection of a sufficient dose of hematoporphyrin to sensitize the animals to sunlight should the entire body be exposed. In all cases the bodies of the animals, however, were protected from the light, and only the blood circulating through the glass tube allowed to come in contact with the rays of light.

The results of this experiment, as summarized in Table II, show that in only one instance, Cat 20, did a greater fall of arterial pressure occur than took place in the normal animals receiving no injection of hematoporphyrin. In one case eosin, which has sensitizing properties similar to those of hematoporphyrin, was injected. None of these animals died during the experiment, but were killed when exposure of the blood to light was discontinued.

Changes Produced in Vitro by Light upon Blood Containing Hematoporphyrin.

Though no differences could be elicited between normal animals and those injected with a sensitizing dose of hematoporphyrin by exposing the blood flowing through a glass tube to strong light, it was not felt that this experiment adequately ruled out the possibility that the striking changes occurring in sensitized animals upon exposure of the body surface to sunlight, might be associated with changes taking place within the blood itself. Accordingly a series of experi-

ments was performed to test the direct effect of hematoporphyrin and sunlight upon blood *in vitro*.

Blood was obtained from both cats and dogs either by venipuncture or directly from the heart and mixed with a small amount of heparin or potassium oxalate to prevent clotting. Each sample was divided into three equal parts. Hematoporphyrin (0.002 gm. to 5 cc. of blood) dissolved in normal saline and a few drops of N/10 sodium hydroxide were added to two of the tubes, while to the third tube only similar amounts of saline and N/10 sodium hydroxide were added. Tubes 1 and 3 were exposed either to sunlight or to the well cooled rays of the 1000 watt carbon arc. Tube 2 was always kept in the dark. The blood in Tube 1, containing hematoporphyrin, invariably changed to a very dark, brownish red color upon

TABLE III.

Changes in the Oxygen Capacity and Content of Blood Produced in Vitro by Addition of Hematoporphyrin and Exposure to Sunlight.

Tube 1		Tube 2		Tube 3	
Blood + hematoporphyrin Exposed to sunlight		Blood + hematoporphyrin Kept in dark		Blood + hematoporphyrin Exposed to sunlight	
O_2 capacity	O_2 content	O_2 capacity	O_2 content	O_2 capacity	O_2 content
vol. %	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*
18.1	6.5	18.0	15.7	18.1	16.5
19.0	6.2	17.0	12.0	17.1	16.8
17.0	2.0	17.5	12.0	17.5	12.0
	3.4		13.0		13.4

exposure to light, the change becoming more marked as the exposure was prolonged. The blood in Tube 2, containing an equal amount of hematoporphyrin, but kept in the dark, showed no change of color. Tube 3, which contained no hematoporphyrin, did not show any change of color even after prolonged exposure to bright sunlight.

It is clear, therefore, that blood to which hematoporphyrin has been added changes to a dark, brownish red color upon exposure to sunlight, and that no such change occurs if such blood is kept in the dark. In the attempt to learn the reason for the striking change of color produced by sunlight in blood containing hematoporphyrin, the oxygen and carbon dioxide contents, as well the oxygen capacity of the three varieties of blood mentioned above were determined by the methods of Van Slyke (Tables III and IV).

From these two tables it will be seen that a marked diminution of oxygen content and increase of carbon dioxide content regularly occurred in the blood of Tube 1, which contained hematoporphyrin and had assumed a dark brownish red color upon exposure to sunlight. The oxygen and carbon dioxide contents of Tube 2, containing blood and hematoporphyrin, which was kept in the dark for the same period that Tube 1 was exposed to sunlight, remained essentially the same as Tube 3, which contained only normal blood without hematoporphyrin, but which was exposed to sunlight along with Tube 1. No changes in color were ever noted in Tubes 2 and 3. The oxygen capacity of all three samples of blood was always approximately the same. Exposure

TABLE IV.

Changes in the Carbon Dioxide Content of Blood Produced in Vitro by Addition of Hematoporphyrin and Exposure to Sunlight.

Tube 1	Tube 2	Tube 3
Blood + hematoporphyrin Exposed to sunlight	Blood + hematoporphyrin Kept in dark	Blood + hematoporphyrin Exposed to sunlight
Carbon dioxide content	Carbon dioxide content	Carbon dioxide content
vol. %	*vol. %*	*vol. %*
32.1	20.8	19.0
28.8	20.8	17.8
25.0	16.8	18.8

of the surface of the blood in the test-tube to the air did not appear to influence the character of the changes regularly observed to occur in Tube 1. This was demonstrated by covering the surface of the blood in all three tubes with a layer of paraffin oil in about half of the experiments.

Causes for Change in Color of Blood Containing Hematoporphyrin When Exposed to Sunlight.

It seems clear that the diminution of oxygen and increase of carbon dioxide content, which has been shown to take place in blood containing hematoporphyrin when exposed to light *in vitro*, are the principal factors which account for the alteration of color of such blood taking place under these conditions. Such qualitative changes, however,

could hardly be held responsible for the distinct brownish tinge which invariably accompanies the darkening in color of such blood.

It was suspected that the brownish color might be due to the presence of methemoglobin, but this was quickly shown to be untrue by repeated spectroscopic examinations of different samples of blood in which the characteristic color changes had occurred. In no instance could a trace of methemoglobin be detected.

TABLE V.

Resistance of New Blood Cells.

Per cent of NaCl in the test-tubes....	0.28	0.30	0.32	0.34	0.36	0.38	0.40	0.42	0.44	0.46	0.48	0.50
Test-Tubes A	+++	+++	+++	+++	+++	+++	++	++	++	+	+	±
containing	+++	+++	+++	+++	++	++	++	+	+	+	±	±
blood and hematoporphyrin exposed to light	+++	+++	+++	++	++	++	++	+	±	±	±	−
Test-Tubes B containing blood	+++	+++	+++	+++	++	++	++	+	+	±	−	−
and hematoporphyrin kept in dark	+++	+++	+++	++	++	+	+	+	−	−	−	−
	+++	+++	++	++	++	+	+	+	±	−	−	−
Test-Tubes C containing blood	+++	+++	+++	++	++	++	+	+	±	−	−	−
without hematoporphyrin exposed to light	+++	+++	+++	++	++	++	+	+	±	−	−	−
	+++	+++	++	++	+	+	+	+	±	±	−	−

+++ complete hemolysis, ++ marked hemolysis, + hemolysis, ± trace of hemolysis.

After centrifugalization, the plasma of Tubes 1, 2 and 3 showed marked differences in color. That of Tube 1 was always very dark red, of Tube 2 a much lighter red, while the plasma of Tube 3 showed only a reddish tinge. Upon pipetting off the plasma and washing the red corpuscles with normal saline solution, the three tubes were again centrifugalized. The saline of Tube 1 was dark red, that of Tube 2 reddish tinged, while that of Tube 3 was colorless. Upon repeating this process upon the same three samples of red corpuscles,

the saline of Tube 1 was still red though of somewhat lighter shade than before, while the saline of both Tubes 2 and 3 remained colorless.

These findings suggested that an increase in fragility of the red blood corpuscles occurred in blood containing hematoporphyrin when exposed to sunlight *in vitro*. Fragility tests were accordingly performed upon the blood of three animals (guinea pigs) to test this hypothesis. Each sample of blood was divided into three equal parts and subjected to the same procedures as outlined above for Tubes 1, 2 and 3. The results of this experiment are summarized in Table V.

As will be seen from Table V, no appreciable difference in resistance could be demonstrated in the red corpuscles of the blood which contained hematoporphyrin and had been kept in the dark, and those of normal blood to which no hematoporphyrin had been added and which had been exposed to sunlight. On the other hand, a distinct increase in fragility of the red cells appeared to have taken place in the blood to which hematoporphyrin had been added before exposure to sunlight. It is at present not possible to state the mechanism by which this fragility of red blood cells is brought about by the combined action of hematoporphyrin and sunlight, but it seems clear that this phenomenon plays an important rôle in the staining of the plasma which occurs regularly under the above mentioned conditions.

The brownish tint observed after exposure to sunlight in blood containing hematoporphyrin is probably due largely to changes in the hematoporphyrin itself. When an alkaline solution of this substance is exposed to sunlight in a test-tube, its bright red color soon changes to a dark, brownish red. This change occurs either in an open test-tube, or when the solution is covered with a layer of paraffin oil to protect it from the air. Such a brownish red solution shows a slight spectroscopic difference from alkaline hematoporphyrin kept in the dark, an extra line appearing in the spectrum between *b* and *c*. Its physiological action, however, remains unaltered. The minimal lethal dose for mice, 0.015 gm., and the amount necessary to fatally sensitize mice to sunlight, 0.005 gm., are the same as for alkaline hematoporphyrin in which this change of color has not occurred.

The Blood Pressure in Hematoporphyrin Shock.

The blood pressure in the carotid artery of cats, under ether anesthesia, was recorded before, during and after the injection of sensitiz-

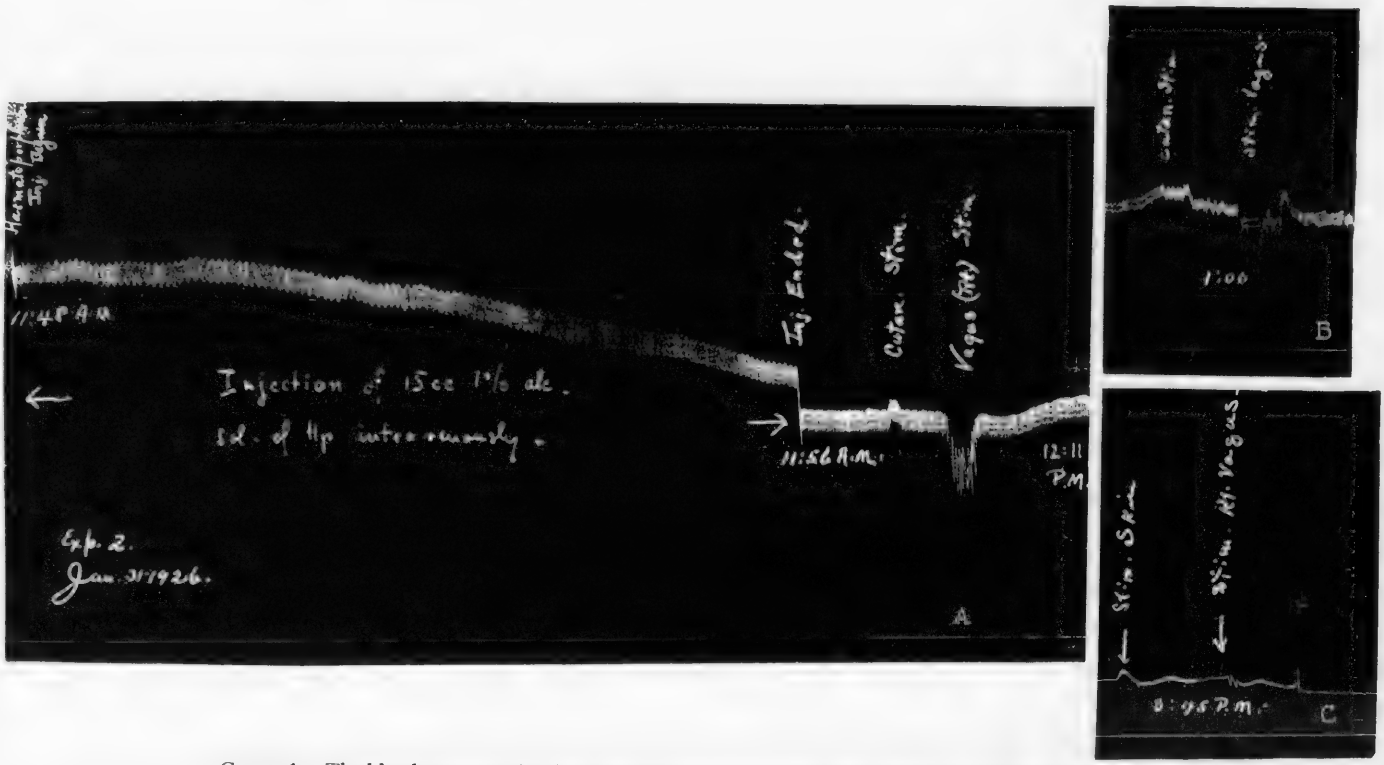

CHART 1. The blood pressure after injection of hematoporphyrin and exposure of the body to sunlight.

ing doses of hematoporphyrin and exposure of the animals to direct sunlight. The crystalline hematoporphyrin was always dissolved in 1 to 2 per cent sodium bicarbonate, and 0.1 gm. of hematoporphyrin per kilo of body weight injected intravenously or subcutaneously.

TABLE VI.

Effect of Exposure to Sunlight upon the Arterial Blood Pressure of Cats Sensitized with Hematoporphyrin.

Animal No.	Date	Length of time of exposure	Exposure	Mean blood pressure		Fall in blood pressure	Remarks
				Before exposure to light	Just before death		
			min.	*mm. Hg*	*mm. Hg*	*mm. Hg*	
Cat 1 (15,508 gm.)	Jan. 17	12.30 p.m.– 1.58 p.m.	88	150	40	110	Intraven. inj. 15 cc. 1% hematoporphyrin
Cat 2 (2,650 gm.)	Jan. 31	11.48 a.m.– 1.06 p.m.	78	120	50	70	Intraven. inj. 20 cc. 1% hematoporphyrin
Cat 3 (2,065 gm.)	June 16	10.10 a.m.–10.45 a.m.	35	120	40	80	Subcut. inj. 25 cc. 1% hematoporphyrin
Cat 25 (1,800 gm.)	May 10	11.00 a.m.–12.15 p.m.	75	120	40	80	Subcut. inj. 25 cc. 0.8% hematoporphyrin

After exposure of such an animal to direct sunlight, the respiration soon becomes more rapid, and the blood pressure falls steadily. After 30 to 60 minutes the respiration has generally become very deep, slow, irregular and labored. At this point the animal is in sufficiently deep coma for the anesthetic to be removed, and after a widely variable length of time (a few minutes to an hour or more) dies. The mean arterial pressure falls rapidly for a time, until the animal is in coma,

and then continues to fall gradually, reaching 40 or 50 mm. just before death (Table VI). The rapidity of the appearance of coma and the fall of blood pressure seems to be directly proportional to the intensity of light and the amount of hematoporphyrin injected. Respiration and heart beat cease simultaneously. Typical blood pressure responses to vagal and cutaneous stimuli were obtained throughout the experiment (Chart 1).

TABLE VII.

Effect Produced upon the Carbon Dioxide-Combining Power, Oxygen Content and Oxygen Capacity of the Blood of Guinea Pigs by Exposure of the Animals to Sunlight after Injection of Hematoporphyrin.

Normal animal exposed to sunlight			Animal injected with hematoporphyrin and kept in dark			Animal injected with hematoporphyrin and exposed to sunlight		
Carbon dioxide-combining power	Oxygen content	Oxygen capacity	Carbon dioxide-combining power	Oxygen content	Oxygen capacity	Carbon dioxide-combining power	Oxygen content	Oxygen capacity
vol. %	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*
47.5	14.0	19.5	48.5	12.9	18.5	16.6	6.5	19.2
40.9	12.6	18.7	37.5	11.5	18.9	19.5	10.5	19.6
44.7	11.8	18.8	38.5	13.7	19.1	18.3	6.9	18.8
54.1						20.2	9.0	
						19.8		

Chemical Changes in the Blood of Animals during Hematoporphyrin Shock.

The following experiments, which were performed upon guinea pigs, were repetitions *in vivo* of the experiments performed *in vitro* upon mixtures of blood and hematoporphyrin already described.

Three animals were used in each experiment. Two of them were injected with a fatal, sensitizing dose of hematoporphyrin. One of these was kept in the dark, while the other, along with the third guinea pig, which had received no hematoporphyrin, was exposed to direct sunlight. Neither the injected animal kept in the dark, nor the normal guinea pig which was exposed to sunlight, showed any alterations in behavior, but the animal which had received hematoporphyrin and was then exposed to sunlight developed the characteristic symptoms of shock as produced under these conditions, and soon died.

Just before the death of this last animal, blood was drawn in a syringe from the right ventricles of the hearts of all three guinea pigs, and immediately placed

under paraffin oil without coming in contact with the air, coagulation being prevented by potassium oxalate mixed with the blood in the syringe. The carbon dioxide-combining power, as well as the oxygen content and oxygen capacity of each of these samples of blood, was determined.

In Table VII, it will be seen that there was little or no difference in the carbon dioxide-combining power of the blood of normal guinea pigs exposed to sunlight and that of animals injected with a fatal sensitizing dose of hematoporphyrin and kept in the dark. Those animals which had received an equal dose of hematoporphyrin and then been exposed to sunlight showed a marked reduction in carbon dioxide-combining power of the blood. No significant difference was found in the

TABLE VIII.

Effect Produced upon the Non-Protein Nitrogen, Creatinine and Sugar of the Blood of Guinea Pigs by Exposure of the Animals to Sunlight after Injection of Hematoporphyrin.

Normal animal exposed to light			Animal injected with hematoporphyrin and kept in dark			Animal injected with hematoporphyrin and exposed to light		
Creatinine	Sugar	N.P.N.	Creatinine	Sugar	N.P.N.	Creatinine	Sugar	N.P.N.
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
2.0	200	27	2.5	160	33	2.3	250	26
1.5	181	33	2.5	166	31	3.0	285	31
1.3	154	32	2.7	285	37.5	2.5	166	35
2.1	222	31.6				2.3	106	42.8
3.0	200	46.1				1.5		
		37.6						

oxygen content of the bloods of normal guinea pigs exposed to sunlight, and those injected with hematoporphyrin and kept in the dark. Guinea pigs injected with hematoporphyrin and exposed to sunlight showed a marked diminution of oxygen in their blood. No change could be demonstrated in the oxygen capacity of blood from these three types of animals.

The non-protein nitrogen, sugar and creatinine of the blood taken from these three groups of animals were determined, but no significant alterations of the amounts of these substances under any of the experimental conditions were found. The results of these studies are summarized in Table VIII.

The red corpuscles and the leucocytes were counted in cats before the injection of hematoporphyrin, and during all stages of shock experimentally produced by this substance. The results of these counts have corroborated the observations of other workers. The red blood cells, which averaged about 8,800,000 per c.mm. in normal cats, showed no significant change during the entire period, which began with the injection of hematoporphyrin and ended with death. The leucocytes, which averaged about 6,000 per c.mm. in normal animals, gradually increased during the experiment to 15,500 per c. mm., and, just before death, rather quickly fell to 4,500 per c.mm. The body temperature was always observed to fall gradually during the development of hematoporphyrin shock. In spite of the fact that the heat of the sunlight was often very intense, the rectal temperature often reached 28–30°C. just before death.

SUMMARY.

The results of these observations may be briefly summarized as follows:

Feeding of hematoporphyrin to white mice over long periods of time produced no apparent changes in these animals and had no effect upon their sensitivity to light.

Albino and slightly pigmented mice and rats injected with hematoporphyrin were protected from the rays of the sun by staining them a blue-black color with Verhoeff's hematoxylin. The dioxyphenylalanine (Dopa) reaction revealed no changes in the cutaneous pigment of animals injected with hematoporphyrin and exposed to sunlight, kept in the dark or diffused daylight. It was therefore assumed that the natural pigment of the skin plays only a physical rôle in protecting animals injected with hematoporphyrin from sunlight.

Exposure to sunlight of only the intestine and mesentery of a cat under ether anesthesia, which had been injected with hematoporphyrin, was followed by death of the animal.

Repeated injections into white mice of large amounts of blood from guinea pigs in hematoporphyrin shock failed to produce symptoms of hematoporphyrin shock. In a parabiosis experiment, one of a pair of white rats promptly developed characteristic symptoms and died

when injected with hematoporphyrin and exposed to sunlight, while the other animal, which was protected from light, but whose circulation had been demonstrated to connect freely with that of its partner, showed no changes during the entire procedure. It has, therefore, been impossible, so far, to demonstrate any substance present in the blood of animals in hematoporphyrin shock which is capable of reproducing this condition in other animals when introduced into the circulation.

Injection of hematoporphyrin followed by exposure of the entire animal to sunlight has been found to produce physiological changes in cats similar to those observed in traumatic shock. There promptly occurred a rapid fall of blood pressure to a very low level and marked lowering of body temperature. The venous blood was found to be poor in oxygen, rich in carbon dioxide and to show low carbon dioxide-combining power. The respiration, which first was accelerated, later on became deep and irregular. The reflexes and typical blood pressure responses to cutaneous and vagal stimulation could always be obtained until death.

Marked diminution of oxygen and increase of carbon dioxide content were found to occur in mixtures of blood and hematoporphyrin exposed *in vitro* to sunlight. These changes in the blood, identical with those occurring *in vivo* during hematoporphyrin shock, support Gaffron's views regarding the effect produced by the combined action of hematoporphyrin and light, but do not further elucidate the nature of the manner in which such alterations take place.

Unsuccessful attempts were made to produce, in both cats and dogs, physiological changes similar to those observed in hematoporphyrin shock by exposing only the blood flowing through a quartz glass cannula, connecting the femoral artery and vein, to strong arclight and sunlight. In another series of animals, which were first injected with hematoporphyrin, exposure of the circulating blood alone to arclight or sunlight did not produce hematoporphyrin shock, although the blood pressure did fall to an unusually low level in one instance.

No changes were found to occur in the amount of non-protein nitrogen, sugar or creatinine of the blood of animals in hematoporphyrin shock.

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RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

I. The Influence of Vaccinia on the Reaction to Infection with Experimental Syphilis.

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The existence of two infectious diseases concurrently in the same individual is now well recognized as not infrequent, and it has been pointed out, among other interesting observations, that the symptoms of each disease may be more severe than when either occurs alone. Probably the infections which most often develop concomitantly or approximately so, are the acute infections of childhood, as for example, scarlet fever and diphtheria, or measles and diphtheria. In other infectious conditions, an intercurrent or complicating infection is a relatively common occurrence, and again, in such circumstances there may be an intensification of symptoms of one or both diseases, as for instance in tuberculosis and syphilis, or measles and tuberculosis. The unfavorable effect of influenza on certain patients with syphilis was shown during the epidemic of 1918 and 1919 in which there were many examples of an abrupt and serious change in a previously mild syphilis following an attack of influenza. On the other hand, a pre-existing disease may be favorably influenced for a longer or shorter time by an intercurrent infectious process, as for example, certain types of psychoses including general paresis, or even cases of malignant disease.

No satisfactory explanation of the various effects observed in these circumstances has been made, for the lack of accurate information regarding their precise nature has not permitted any but the most general statements. It is surprising, in view of the modification of symptoms which may occur and the significance which such alterations undoubtedly have from the standpoint of the host's reaction to disease, that the subject has received comparatively little experimental investigation.

Our interest in concomitant diseases arose first during our study of experimental syphilis and yaws, particularly with reference to superinoculation and crossed inoculation, and later, in connection with a transplantable malignant neoplasm of the rabbit which developed in a syphilitic animal. More recently our attention was again directed to this subject as a field for experimental investigation from observations of a strain of *Treponema pallidum* obtained from a rabbit found to be also infected with vaccine virus. It became evident that this virus was being transferred with the syphilitic inocula. The syphilis which developed in rabbits inoculated with this material was unusually mild, and this finding was wholly unexpected since this particular strain has been carried in rabbits for many years and is reported to be capable of inducing a severe disease. The possibility that the accompanying vaccinal infection contributed to these unforeseen results was naturally considered, but the acceptance of such an explanation obviously required experimental evidence obtained under properly controlled conditions. To this end, several series of experiments were undertaken in which was studied the influence of a vaccinal infection upon the reaction to syphilis induced by a strain of *T. pallidum* uncontaminated with vaccine virus. An extension of the investigation comprised experiments dealing with the reaction to *T. pallidum* of rabbits immune to vaccine virus.

The present paper contains the results of experiments in which one of the two commonly used routes of syphilitic inoculation was employed, namely, the intratesticular; the vaccinal inoculations were made intracutaneously on the side of the body. Subsequent papers contain the results of experiments in which *T. pallidum* and vaccine virus were inoculated in the same testicle, with observations on the syphilitic reaction of rabbits immune to vaccine virus and with the results of the intracutaneous route of both syphilitic and vaccinal inoculations. A summary of the results obtained has already appeared (1).

EXPERIMENTAL.

Materials and Method.

Three experiments are reported on the course of syphilitic infection in rabbits inoculated simultaneously with vaccine virus and with the Nichols strain of

T. pallidum. For comparison, other series were inoculated only with *T. pallidum*. The Noguchi strain of vaccine virus which was used was obtained from a fresh testicular lesion; its virulence was controlled by inoculation in normal rabbits using the intracutaneous and intratesticular routes as well as applications to scarified skin areas.

The experimental animals were inoculated intracutaneously (0.2 cc.) and on a scarified skin area (0.2 cc.) with vaccine virus and immediately thereafter with *T. pallidum* injected into the right testicle. The emulsions used were prepared from actively growing testicular lesions and contained from 1 to 3 spirochetes to the microscopic field; each animal received 0.2 cc.

The rabbits employed were young adult male animals approximately 8 months of age. They were placed in individual cages and were divided into 2 groups as nearly comparable as possible with respect to age, breed and weight. In each experiment, the control group, that is the group inoculated only with *T. pallidum*, comprised 10 rabbits; in the first and second experiments there were 5, and in the last, 10 animals in the vaccinated group.

The dates of inoculations were as follows: November 10, 1926, January 13 and February 14, 1927. The periods of observation were from 3 to 5 months, but for the purpose of comparing the results of the 3 experiments one with another, the data obtained during the first 3 months following inoculation have been used.

In these experiments special attention has been given to the time and frequency of occurrence and to the duration of successive phases of the reaction to syphilitic infection with a view to reducing the comparison of results to as quantitative a basis as possible. The particular conditions chosen for comparison were, (1) the incubation time of primary lesions, (2) the time and frequency of the occurrence of a critical edema in the inoculated testicle, (3) the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), (4) the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones or eyes, (5) the number of foci affected by such lesions, (6) the proportion of animals that showed complete healing of all lesions during the 3 months observation period.

It should be noted, in discussing time relations of the various reactive phenomena, that there is a basic tendency toward the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits. With the Nichols strain of *T. pallidum* as carried in our laboratory, the reaction interval is approximately 2 weeks. In these experiments, as stated above, the Nichols strain was used, but the particular line from which the present substrain, as it may be termed, was derived had been transferred under somewhat different conditions from those employed with the parent strain. It was not known at the time this work was started, however, that the infection induced by this substrain would differ in any essential respect from that of the parent strain, but the results of these and other experiments have shown certain peculiar features of the syphilitic reaction, notably a delay in the development of a metastatic orchitis and an un-

usually early appearance of generalized lesions. These peculiarities in the behavior of the strain must be taken into account in analyzing the results obtained.

The methods employed for recording results require little explanation. The term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions, while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparison of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

Results.

The results of the experiments are recorded in Tables I, II and III and in Text-figs. 1 and 2. It will be noted that the figures given in

TABLE I.

Incidence of the Various Phenomena of the Syphilitic Infection and the Focal Distribution of Generalized Lesions.

Experiment	Animal group	No. of rabbits	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
						Incidence	Focal distribution	Focal distribution
			per cent	*per cent*	*per cent*	*per cent*	*actual*	*relative*
I	C	10	100.0	60.0	100.0	100.0	9.8	9.8
	V V	5	100.0	80.0	100.0	100.0	22.2	22.2
II	C	10	100.0	50.0	90.0	70.0	7.0	4.9
	V V	5	100.0	40.0	80.0	100.0	14.4	14.4
III	C	10	100.0	70.0	90.0	90.0	8.4	7.6
	V V	10	100.0	44.4*	88.8	100.0	16.3	16.3
Mean values	C	30	100.0	60.0	93.3	86.7	8.6	7.4
	V V	20	100.0	52.6	89.5	100.0	17.4	17.4

C = controls; V V = animals inoculated with vaccine virus.

* One animal in this group which developed edema died shortly thereafter; its inclusion would bring the incidence of edema to 50.0 per cent.

Tables I and II and in the text-figures represent group values. In Text-fig. 1, the detailed results are presented graphically in 3 charts in order to show the entire sequence of events from the development of the first or primary lesions to the appearance of the last generalized lesions; the abscissæ represent the time after inoculation in days. These curves also illustrate an important feature of the syphilitic reaction, namely, that while these events occur successively, they also overlap each other to some extent. In order to simplify the reading of the charts, a division of the curves has been made at the 35th day

TABLE II.

Mean Time of Occurrence of the Various Phenomena of the Syphilitic Infection as Estimated in Days from the Date of Inoculation.

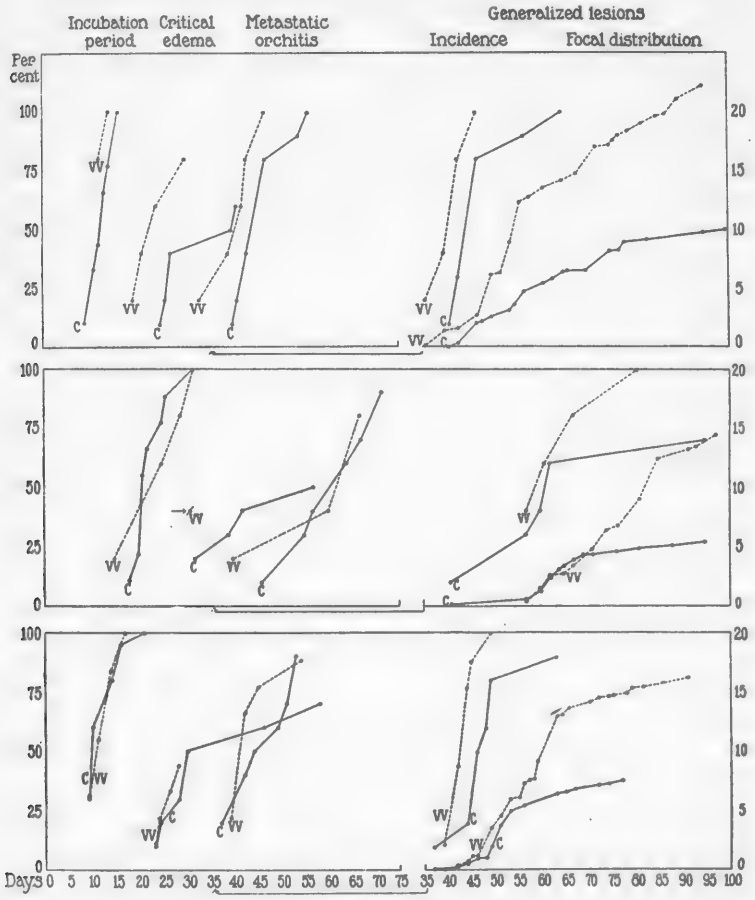
Experiment	Animal group	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					First	Last	Mean of all
		days	*days*	*days*	*days*	*days*	*days*
I	C	12.8	31.0	46.5	48.0	78.8	62.8
	V V	12.4	23.5	40.4	41.2	95.0	62.7
II	C	22.9	40.4	61.8	62.0	80.4	67.4
	V V	23.6	31.0	58.5	63.4	87.4	75.9
III	C	12.2	33.9	45.3	47.6	67.6	55.0
	V V	12.0	25.3	43.0	43.4	80.1	59.6
Mean values	C	15.8	34.7	51.0	51.6	75.1	61.0
	V V	15.7	25.7	45.9	48.1	88.6	64.0

as indicated by arrows, and the curves representing the incidence and focal distribution rates of generalized lesions have been separated from the others. The time of appearance or the distribution of generalized lesions is shown by the curves in Text-fig. 2 in which a time interval of 4 days has been used. Table III contains the total number of generalized lesions. In the following discussion, mean values have been used for the most part since the results of the individual experiments are in essential agreement one with another.

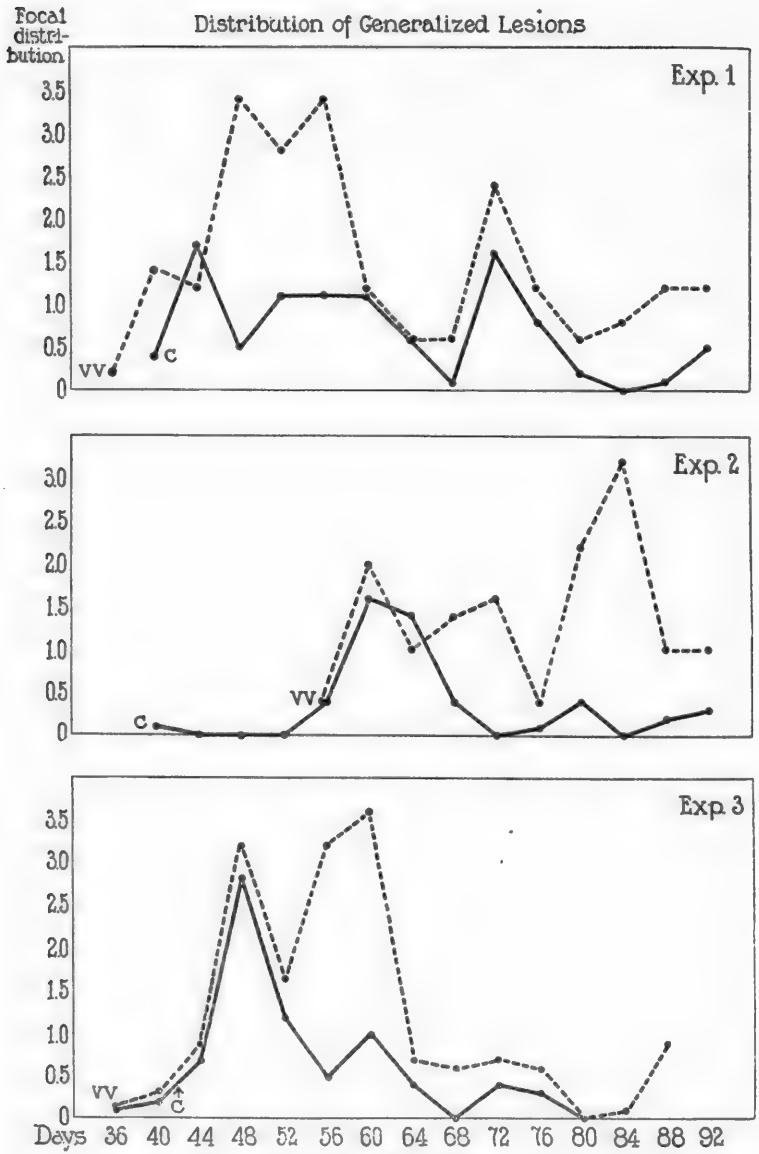
TABLE III.

Time of Appearance of the Generalized Lesions as Estimated from the Date of Inoculation.

Time interval	Experiment I		Experiment II		Experiment III	
	No. of lesions		No. of lesions		No. of lesions	
	C (10 rabbits)	V V (5 rabbits)	C (10 rabbits)	V V (5 rabbits)	C (10 rabbits)	V V (9 rabbits)
days						
35	0	1	0	0	0	0
37	0	0	0	0	1	1
39	0	6	0	0	0	0
41	1	0	1	0	2	3
43	3	1	0	0	3	4
45	0	0	0	0	4	4
47	19	6	0	0	1	0
49	3	17	0	0	9	20
51	0	1	0	0	18	9
53	6	13	0	0	12	13
55	5	16	0	0	5	13
57	11	1	4	2	0	3
59	0	0	7	5	0	14
61	7	6	9	6	0	0
63	4	0	7	0	10	34
65	6	3	3	1	1	5
67	0	3	4	3	3	0
69	1	0	3	0	0	5
71	0	0	1	7	3	4
73	0	11	0	8	1	1
75	16	3	1	0	0	1
77	8	2	0	2	3	1
79	0	2	0	10	0	4
81	0	3	4	11	0	0
83	2	0	0	0	0	1
85	0	3	0	17	0	0
87	0	1	2	0	0	2
89	1	6	0	4	0	0
91	5	6	3	6	0	5
Total	98	111	49	72	76	147


Influence of Concomitant Vaccinal Infection on Reaction to Syphilis
Incubation period
Critical edema
Metastatic orchitis
Generalized lesions
Incidence
Focal distribution
Per cent
Days

TEXT-FIG 1.


Focal distribution
Distribution of Generalized Lesions
Exp. 1
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
VV
C
Exp. 2
3.0
2.5
2.0
1.5
1.0
0.5
0
C
VV
Exp. 3
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
VV
C
Days 36 40 44 48 52 56 60 64 68 72 76 80 84 88 92

TEXT-FIG 2.

DISCUSSION.

The purpose of this investigation was to determine whether a concomitant vaccinal infection would influence the reaction to experimental syphilis. It should be kept in mind that in the experiments reported in the present paper, the inoculation of both viruses was made at the same time but at different sites—the vaccine virus intradermally on the side of the body and *T. pallidum* in one testicle. The results obtained have been analyzed in terms of various reactive phenomena in accordance with the general principles that govern the evolution of syphilitic infection (2, 3) and in the following discussion these phenomena are taken up in the order of their occurrence, so that the picture of the disease as it developed may be more readily visualized.

Incidence of Primary Lesions and Incubation Period.—All rabbits in these experiments developed primary lesions (Table I; Text-fig. 1), and there was practically no difference in the length of the mean incubation period between the vaccinated and control groups, that is 15.7 and 15.8 days (Table II). It will be noted that all incubation periods for the first and third experiments were essentially the same, 12.0, 12.2, 12.4 and 12.8 days, while those of the second were prolonged to 22.9 days in the case of the controls and to 23.6 days for the vaccinated group. As will be seen later, the magnitude of these values are indicative of the general character of the disease that prevailed, that is, it was much less severe in the second than in the first or third experiment.

It is evident from these findings that the concomitant intradermal inoculation of vaccine virus did not influence either the incidence of the primary syphilitic reaction or the time of its development as determined clinically by palpation.

Critical Edema.—While the occurrence of a critical edema in an inoculated testicle or in association with other syphilitic lesions is a variable phenomenon, it has an important significance as indicating an intense reaction; it usually marks the end of a local reaction, either temporary or final.

The mean values of the incidence of edema in the inoculated testicle were essentially the same for the controls and the vaccinated animals,

that is 60.0 and 52.6 per cent respectively, but the incidence in individual experiments was variable (Table I and Text-fig. 1). In the first experiment in which the disease was very severe there was a higher frequency in the vaccinated than in the control groups, but this order was reversed in the third experiment with a disease of somewhat less severity, while in the second in which a relatively mild syphilis prevailed, there was little difference between the 2 groups.

The time at which edema occurred, however, indicates very clearly the more prompt reaction of the vaccinated animals as shown by the mean values calculated from the time of inoculation, that is 28.6 for the vaccinated and 34.7 days for the control group (Table II). The results of individual experiments were similar, the difference in time between vaccinated and control groups being 7.5, 9.4 and 8.6 days respectively. In addition, it should be pointed out that there was a closer agreement in the time of development of edema among individual animals of each vaccinated group than among the controls as shown by the curves of Text-fig. 1. In the control groups, there were 2 rabbits each in the first and third and 1 in the second experiment in which an edema of the inoculated testicle was considerably delayed. And although the group and mean time values are obviously influenced by these particular animals, still their omission does not disturb the order of group values as shown by the following figures:

Experiment	"Corrected" controls		Vaccinated	
	No. of rabbits	Edema	No. of rabbits	Edema
		days		*days*
I	4	26.3	4	23.5
II	4	36.3	2	31.0
III	5	26.8	4	25.3
Mean values	13	29.5	10	25.7

An idea of the progress of the reaction is also obtained by estimating the time of appearance of edema from the incubation period of the primary lesion instead of from the date of inoculation, as has been done in the table at top of following page.

From Incubation.

Experiment	All controls	"Corrected" controls	All vaccinated rabbits
	days	*days*	*days*
I	18.2	13.5	11.1
II	17.5	13.4	7.4
III	21.7	14.6	13.3
Mean values	18.9	13.7	10.0

A comparison of these values brings out very clearly the fact that there was a more prompt reaction to the syphilitic infection in the vaccinated than in the control rabbits irrespective of whether all controls are included in the comparison or only those animals in which the development of edema was not delayed.

As has already been mentioned, the usual time interval between the occurrence of successive reactions with highly virulent strains of *T. pallidum* is 2 weeks. The above table shows that with respect to the interval between the incubation period of the primary lesion and the critical edema, the majority of controls conformed to this value (13.7 days), while it was somewhat lengthened in the case of all controls (18.9 days). With the vaccinated animals, on the other hand, this interval was shortened to the unusually short time of 10.0 days.

The essential point to be remembered with respect to the phenomenon of critical edema is the earlier time in which this reaction occurred in the vaccinated groups as compared with the controls of these experiments, bearing in mind that this interval in the controls was not shorter than that usually seen in rabbits infected with the Nichols strain.

Metastatic Orchitis.—One of the most characteristic and regular phenomena of the syphilitic reaction is the occurrence of a metastatic orchitis in the uninoculated testicle which with a disease of well marked severity develops approximately 6 weeks after inoculation.

The incidence of a metastatic orchitis in the control and vaccinated groups of the present experiments was the same, as is shown in Table

I and Text-fig. 1. It occurred in all animals of both groups in the first experiment in which a disease of marked severity prevailed, while it was not detected in 1 animal each of the vaccinated and control groups of the second and third experiments in which the disease was less pronounced.

With respect to the time at which a metastatic orchitis developed, the vaccinated groups showed a more prompt response (Table II). The mean values reckoned from the day of inoculation were 51.0 days for the controls and 45.9 days for the vaccinated animals. These mean values are higher than those ordinarily seen with strains of high virulence, owing largely to the prolonged reaction time observed in the second experiment. But in this experiment as well as in the others, an orchitis of the uninoculated testicle occurred earlier in the vaccinated than in the control groups, the difference in time being 3.1 days in the second, 6.1 days in the first and 2.3 days in the third experiment. Moreover, as shown in Text-fig. 1 it uniformly occurred sooner in the vaccinated group than in the controls in the first experiment, and so too with most of the animals in the third experiment. In the second experiment, however, as might be expected with a mild disease, the lesions developed irregularly with no definitely defined group difference.

The results bearing on the development of a metastatic orchitis with respect to the time values of the previous reactions, that is to say, the incubation period of the primary lesion and the critical edema are shown by the following figures:

From the Incubation Period.

Experiment	Controls	Vaccinated rabbits
	days	*days*
I	33.3	28.0
II	38.9	34.9
III	33.1	31.0
Mean values	35.2	30.2

From the Critical Edema.

Experiment	Controls	"Corrected" controls	Vaccinated rabbits
	days	*days*	*days*
I	15.2	20.2	16.9
II	21.4	25.5	27.5
III	11.4	18.5	17.7
Mean values	16.3	21.7	20.3

With the Nichols strain, the interval between the development of a metastatic orchitis and the incubation period of the primary orchitis is usually from 4 to 5 weeks. In the case of the first and third vaccinated groups as well as with the mean value for the 3 groups this interval was actually or approximately 4 weeks, while the average intervals for the individual control groups as well as the mean value were approximately a week longer. Comparisons based upon the interval between the occurrence of the critical edema and the development of the metastatic orchitis are less satisfactory with group than with individual animals because of the variability in the incidence and time of occurrence of this phenomenon. In the present instance, if the comparison includes all control animals that developed edema, the interval is considerably shortened, owing to the delayed edema of 5 rabbits, and the resulting mean and individual group values are consequently smaller than the corresponding ones for the vaccinated animals. But with the omission of these 5 rabbits ("corrected" controls), this interval between edema and metastatic orchitis is slightly shorter in the vaccinated than in the control groups of the first and third experiments. It is slightly longer in the second experiment, a discrepancy which may be explained by the difference in the number of animals in the control (corrected) group which developed edema—that is 4 as contrasted with 2 in the vaccinated group. Such small differences, however, are of interest only in that they are in agreement with other results.

From the above analyses of the results obtained in connection with the incubation period of the metastatic orchitis with reference to the time of inoculation as well as from the time relations between its oc-

currence and that of preceding reactive phenomena, it is clear that the reaction to the syphilitic infection as measured by the phenomena of a metastatic orchitis was more prompt in rabbits which were vaccinated with vaccine virus coincidently with the inoculation of *T. pallidum* than in control animals. It will be recalled that a similar result was obtained in connection with the preceding phenomenon of a critical edema.

Generalized Lesions.—The phenomenon of generalized lesions is the most indicative single basis for estimating the character of the reaction to syphilitic infection. The occurrence or non-occurrence of generalized lesions, the time of their appearance, and the duration of the period of active development, their number, extent, character and persistence are essential features of this phase of the disease which can be employed in analyzing its character. The disease exhibits a well recognized variability in all these respects, but it is usually comparatively constant under a given set of conditions.

In the present experiments, a large number of control animals developed generalized lesions, 86.7 per cent, but this was surpassed by the vaccinated groups with the unusually high incidence of 100.0 per cent (Table I). As has been mentioned before, the disease in the first experiment was very severe, in the second, comparatively mild, while in the third it was quite severe, and these differences are indicated by the incidence of generalized lesions in the control groups, namely, I, 100.0 per cent; II, 70.0 per cent; III, 90.0 per cent (Table I), but irrespective of these variations, generalized lesions occurred in every vaccinated rabbit.

In addition, an extremely large number of lesions developed in the vaccinated animals, the mean actual and relative distribution rates for the vaccinated groups being 17.4 as compared with control rates of 8.6 and 7.0 (Table I). Results of similar orders of magnitude were obtained in individual experiments. The extent of the differences between the vaccinated and control groups in this respect is better appreciated by estimating the focal distribution rates of the vaccinated groups in percentage terms of control values, as has been done in the table at top of following page.

Experiment	Actual rate of control values	Relative rate of control values
	per cent	*per cent*
I	+122.4	+122.4
II	+132.9	+230.6
III	+94.1	+114.5
Mean values	+102.3	+148.6

These figures demonstrate in a striking manner that the so called generalized phase of the syphilitic infection as measured by the actual number of lesions detected was much more pronounced in the animals which received an intracutaneous injection of vaccine virus at the time of intratesticular inoculation with *T. pallidum* than in the controls.

The time at which generalized lesions developed is another point of comparison in which the vaccinated rabbits differed from the controls. Reckoned from the day of inoculation, as has been done in the analyses summarized in Table II, the mean time of the appearance of the first generalized lesions in the vaccinated groups antedated that of the controls by 3.5 days, while the mean time of the last lesions to develop was 14.5 days later in the vaccinated than in the control animals. These differences as well as those obtaining in individual experiments are shown in the following table in which a minus or a plus sign indicates a shorter or a longer time than that for the controls.

Difference in Time of Appearance of Generalized Lesions of Vaccinated Groups as Compared with Controls.

Experiment	First lesion	Last lesion
	days	*days*
I	−6.8	+17.2
II	+1.4 (−2.1)*	+7.0
III	−4.2	+12.5
Mean values	−3.5 (−3.9)*	+13.5

* Values obtained by omitting one control animal with a single precociously early lesion.

Although the differences with respect to the last lesions are more striking than those for the first, still the first differences demonstrate the significant fact that the reaction time of the vaccinated groups in regard to another phenomenon of the syphilitic infection, that is to say, the development of generalized lesions, continued to be in advance of that of normal animals as was seen with the earlier phenomena of critical edema and metastatic orchitis. It should be mentioned that the corrected figure for the time difference in the second experiment is a fairer expression of the group picture than the uncorrected, as will be seen by referring to Text-fig. 1. That the severity of the disease prevailing in different experiments is an important factor in influencing the extent or range of the results obtained is well illustrated by the relative magnitudes of the values for the first and last lesions in the above table, the highest values being found in the first experiment in which the most severe disease prevailed, while the lowest occurred in the second with a comparatively mild disease.

From what has been said regarding the time of appearance of the first and last generalized lesions, it is obvious that the period of active development of this important phase of the disease was considerably longer in the case of the rabbits which received vaccine virus. The extent of these differences is strikingly brought out by the following comparison:

Period of Active Development of Generalized Lesions.

Experiments	Controls	Vaccinated	Increase of vaccinated groups		
	days	*days*	*days*		*per cent*
I	30.8	53.8	+23.0	or	74.8
II	18.4	24.0	+ 5.6	or	30.5
	(14.9)*		(+9.1)*	or	(61.0)*
III	20.0	36.7	+16.7	or	83.5
Mean values	23.5	40.5	+17.0	or	72.3
	(23.1)*		(+17.4)*	or	(75.3)*

* These values were obtained by omitting 1 animal with a precocious lesion, as explained in the foregoing text.

These figures show that in each experiment the period of active development of generalized lesions was much longer in the vaccinated

than in the control animals. The percentage increases in time of the first and third experiments, 74.8 and 83.5 per cent, are of the same order of magnitude as the mean value of 72.3 per cent. In the case of the second experiment, the increase of 30.5 per cent is considerably smaller, but the corrected figure of 61.0 per cent is comparable to the others; and as has already been mentioned, the corrected figure is more representative of the group as a whole.

In round numbers, the mean value of the duration of activity as regards generalized manifestations was 3 weeks in the case of the controls and 6 weeks in the case of the vaccinated animals. There was comparatively little difference between the 2 groups, however, in regard to the mean time of appearance for *all* generalized lesions reckoned from the day of inoculation, that is 61 and 64 days for the controls and vaccinated groups respectively (Table II); and a similar result is obtained if the interval is estimated from the incubation time of the primary orchitis. But if the comparison is made from the time of the reactive phenomenon immediately preceding the appearance of generalized lesions, namely, the development of a metastatic orchitis, the difference between the 2 groups is well shown. The results of these 2 analyses appear in the following tabulations:

From Primary Orchitis to Mean Time for All Generalized Lesions.

Experiment	Controls	Vaccinated
	days	*days*
I	50.0	50.3
II	44.5	52.1
III	42.8	47.6
Mean	45.8	50.0

From Metastatic Orchitis to Mean Time for All Generalized Lesions.

Experiment	Controls	Vaccinated
	days	*days*
I	16.3	22.3
II	5.6	17.2
III	9.7	16.6
Mean	10.5	18.7

Both these comparisons of time reactions show the existence of a longer interval between the date of the previous reactive phenomena and the mean time of appearance of all lesions in the case of the vaccinated animals as compared with the controls, but the difference is greater with respect to the phenomenon immediately preceding the development of generalized lesions, namely, the metastatic orchitis. From the various analyses of the results already presented, it is evident that this second comparison is more indicative of the general character of the disease prevailing in the 2 groups than in the first, and it illustrates one of the biological principles governing syphilitic infection, namely, that the character of any reaction is closely connected and, indeed, is particularly dependent upon the nature of the preceding reaction. In addition, the foregoing tables bring out the fact that, as regards these time relations, the vaccinated groups were more uniform than the controls, and this was to be expected under the conditions of relatively uniform disease severity prevailing in the vaccinated animals.

Neither of these comparisons involving the mean time of appearance for *all* generalized lesions, however, shows a marked divergence between control and vaccinated groups, and this is largely due to the small difference between these mean values. The differences that exist in the actual distribution of lesions in the vaccinated as compared with the control animals, however, are shown in the distribution curves of the times at which new lesions appeared (Text-fig. 2). A time interval of 4 days has been chosen in plotting these curves to obviate some of the irregularities associated with shorter periods, as shown in Table III which lists the total number of lesions in 2 day intervals. The relative rather than the actual number of lesions has been used because of the difference in number of animals in the groups of the first 2 experiments, so that in addition to the time distribution of lesions, the height of the curves gives an idea of the severity of the disease as measured by the numerical distribution of lesions in the order of their appearance.

Although the 3 sets of curves show certain dissimilar features due largely to the different character of the disease prevailing in individual experiments, those representing the vaccinated animals are in remarkable agreement as regards their general levels which are much higher

throughout than the control curves. For the most part, the fluctuations of the two curves in the first and third experiments, in which the disease was pronounced, occur in the same time interval, showing that in general the reaction of the vaccinated group had not been altered as regards the time of appearance of generalized lesions, but the much greater height of the curve for the vaccinated animals shows that the intensity of the reaction was profoundly affected as measured by the number of lesions which developed. There were three outstanding exceptions, however, to the synchronous time reaction of the vaccinated animals as compared with the controls. In the first experiment in which the most severe disease prevailed, the first generalized lesions appeared earlier in the vaccinated than in the control animals. In the second experiment many lesions developed in the vaccinated rabbits during the last 4 weeks of the observation period, while there were extremely few among the controls, and in the third, lesions were developing in the vaccinated animals when the period of observation was discontinued, although no lesions had appeared in the controls for the preceding 2 weeks.

Without analyzing these curves in detail, it is apparent that with a syphilitic disease of varying degrees of severity, it has been possible to increase the numerical distribution of generalized lesions and to prolong the period of active development of lesions over and beyond that of normal animals by means of an intracutaneous inoculation with vaccine virus made at the time of an intratesticular inoculation with *T. pallidum*. The general schedule of the times at which lesions first appeared, however, was not appreciably disturbed except in the experiment in which the disease of the control animals was mild, and this exception should be especially noted. The two curves of the second experiment are similar in their first portion, the peak of both occurring in the 60 day interval. The control curve then falls to the base line (72nd day) and practically continues there, while there are two high peaks in the vaccinated curve occurring in the 72nd and 84th day intervals, and at no time does it reach the base line. Furthermore, the second of these two peaks is higher than the first, showing that a greater number of lesions first appeared late in the course of the disease. Such an occurrence is unusual and indicates that earlier reactions were not sufficient to prevent the development of additional

and numerous late lesions; in the case of the control animals, as shown by the curve, an apparently similar early reaction was sufficient. In the third experiment, the second peak of the vaccinated curve closely follows the first, and apparently the reaction associated with such a shower of lesions was sufficient to prevent the later development of numerous lesions, although it should be noted that at its end, the curve rises abruptly to an appreciable height indicating that a number of generalized lesions were continuing to appear. In the first experiment, the curve of the vaccinated animals is a marked exaggeration of the control curve. It will be noted that the peak of the control curve in the 72nd day interval follows a long period (from the 40th day) in which many generalized lesions developed continuously at a fairly uniform rate and that there was not an early and relatively short period of marked eruptive activity which ordinarily would not be followed by successive periods of lesion development.

Recovery.—The last phase of the syphilitic reaction to be considered is that of complete resolution and healing of all lesions within the 3 months period of observation. Under ordinary circumstances, certain rabbits show no lesions by this time; in others residual lesions, usually of the genitalia, remain, while some animals continue to show active generalized manifestations. In the first and second experiments, the resolution of lesions was delayed, owing in one instance to the severity of the infection, and in the other to the late development of lesions and the consequent prolongation of the time of resolution and healing as reckoned from the day of inoculation. The condition of the lesions at the end of the observation period, therefore, has been described as follows: healed, not healed and active. The comparison of the vaccinated and control animals upon this basis gives the results, expressed in per cent, as shown on following page.

These figures show that the vaccinated animals differed markedly from the controls in the final phase of the syphilitic reaction, and furthermore, that the character of the difference was in accord with what was observed in earlier phases of the reaction. In the great majority of vaccinated animals, 89.5 per cent, the reaction to the syphilitic infection had not ceased at the end of the observation period, for lesions not only continued to be active, but as has already been mentioned, fresh lesions were developing in many animals. On the

other hand, only 26.7 per cent of the controls showed a comparable state of lesion activity, the majority of the animals having entered upon the phase of resolution.

Final Condition of the Lesions.

Experiment		Healed	Not healed	Active
		per cent	*per cent*	*per cent*
I	Controls	10.0	50.0	40.0
	Vaccinated			100.0
II	Controls		60.0	40.0
	Vaccinated		20.0	80.0
III	Controls		100.0	
	Vaccinated		11.2	88.8
Mean values	Controls	3.3	70.0	26.7
	Vaccinated		10.5	89.5

At this point, something should be said of the general character of the lesions. It is well recognized that in a group of 5 or 10 rabbits, there is an individual animal variation as regards size, consistency and destructiveness of both genital and generalized lesions, which features are, of course, associated with the duration and degree of active development and persistence of lesions. In the present experiment, variations in these respects occurred in individual rabbits of both the control and the vaccinated groups, but in general, it may be said that both the genital and generalized lesions of the vaccinated animals were larger, more destructive and persisted longer than those of the controls. This difference was particularly striking in the case of scrotal chancres, and of bone and periosteal granulomata.

The disease which was observed in this investigation presented certain peculiar and uncommon features, especially of time reactions and of lesion type, which should be referred to, as they must be kept in mind in appraising the experimental results obtained. For example, the development of the metastatic orchitis was delayed while generalized lesions appeared unusually early, so that the two reactions occurred within a few days of each other instead of being separated by the usual interval of approximately 2 weeks. As a matter of fact,

in approximately one half of the total number of both the control and vaccinated animals, the appearance of generalized lesions preceded the detection of the metastatic orchitis by a few days, or both reactions were observed to occur on the same day as is shown in the following table:

Appearance of Generalized Lesions in Relation to Metastatic Orchitis.

Controls	Earlier	Same day	Total
	per cent	*per cent*	*per cent*
I	40.0	20.0	60.0
II	30.0	10.0	40.0
III	40.0	10.0	50.0
Mean values	36.7	13.3	50.0
Vaccinated			
I	20.0	20.0	40.0
II	20.0		20.0
III	33.3	33.3	66.7
Mean values	31.6	21.1	52.7

These figures, as well as the time intervals between the development of the metastatic orchitis and the appearance of the first generalized lesions (Table II) indicate that an intracutaneous inoculation of vaccine virus coincident with the intratesticular inoculation of *T. pallidum* did not affect this peculiar feature of the disease.

The infection was further characterized by an unusually high preponderance of bone lesions and a relatively low number of skin lesions. Up to a certain point, such a distribution is to be expected, for numerous and severe cutaneous manifestations do not usually develop in animals in which previous bone involvement has been extensive. But this relationship does not obtain in cases of very severe or malignant syphilis which are characterized by numerous extensive and persistent lesions of both bone and skin and frequently of other tissues as well. In the first experiment, there were many instances of syphilis which could be called malignant judging from the character of genital and bone lesions, and in the second, there were several which approached this state, but nevertheless, there were comparatively few cutaneous manifestations, other than those of the

scrota which are not usually included among generalized or secondary lesions. This aspect of the infection is illustrated by the following table:

Relation of Skin and Bone Lesions.

Experiment		Total Relative No.	Bone Relative No.	Skin	
				Relative No.	Per cent of total
I	Controls	9.8	9.1	0.7	7.14
	Vaccinated	22.2	20.0	2.2	9.09
II	Controls	4.9	3.6	1.3	26.53
	Vaccinated	14.4	14.0	0.4	2.77
III	Controls	7.6	7.5	0.1	1.32
	Vaccinated	16.3	15.6	0.7	4.29
Mean values	Controls	7.4	6.7	0.7	9.44
	Vaccinated	17.4	16.4	1.0	5.75

The relatively large proportion of skin lesions in the control animals of the second experiment is apparently out of harmony with the findings in both groups of the first and third series. However, if the values for individual experiments are tabulated in the order of the severity of disease, the situation becomes somewhat clearer:

Experiment	Controls	Vaccinated
	per cent	*per cent*
I	7.14	9.09
III	1.32	4.29
II	26.53	2.77

This arrangement shows that for all vaccinated groups and for the first and third control groups, the proportion of skin to total secondary lesions follows a consistent numerical relation as regards the different levels of disease severity in the 3 experiments, and furthermore, that in the second experiment in which the infection was less marked than in the others, the proportion of skin to total lesions was not disturbed in the vaccinated animals. In the second control group, on the other hand, there was a relatively large number of cutaneous

manifestations but these were preceded by comparatively few bone lesions, a finding which was absent in the corresponding vaccinated group as is shown in the following table:

Relative Number of Bone Lesions.

Experiment	Controls	Vaccinated
I	9.1	20.0
III	7.5	15.6
II	3.6	14.0

It is evident from these figures that the numerical relationships of bone and skin lesions in the second vaccinated and control groups were in harmony with what is known of this phase of syphilitic infection, and the seeming contradiction of the second vaccinated group with respect to the number of skin lesions as compared with the corresponding control group is in reality in accord with the preservation of disease type and severity on the part of the vaccinated animals.

Still other evidence of the influence induced by vaccinal inoculation upon the course of the syphilitic disease is furnished by the distribution curves of generalized lesions (Text-fig. 2). The shape of the curves representing the control groups illustrate the general relations that obtain under ordinary conditions with respect to the numerical distribution of secondary lesions, that is, the early development of numerous lesions is not usually followed by the successive appearance of many others (see curves of Experiments II and III), while severe syphilis is characterized by the continued appearance of lesions for a prolonged period, a fact exemplified in the control group of the first experiment. The curves illustrating the distribution of lesions in all vaccinated groups show that this relationship was interfered with, for despite the large number of lesions that appeared early in the period of generalized manifestations, numerous lesions continued to develop in these animals.

It is evident from the results obtained in these experiments that intracutaneous inoculation with vaccine virus at the time of intratesticular inoculation of *T. pallidum* was associated with a profound disturbance in the syphilitic reaction, particularly as regards the rela-

tive frequency of occurrence of various reactive phenomena, the altered time relationships of these manifestations and in the relative failure on the part of one phenomenon to prevent or delay the development of successive manifestations. As measured by the various criteria selected for comparison, the efficiency of reaction of the vaccinated animals was unquestionably lowered with the result that the infection pursued a severe and uninterruped course.

The coexistence of two or even three and four various infections has been attributed to an "increased susceptibility" on the part of the patient consequent upon the severity of the primary disease and in attempts to explain the gravity of these cases, the rapidity with which the diseases follow one another has been frequently invoked. In the present experiments, the syphilitic and vaccinal inoculations were made simultaneously. It must be remembered though that the reaction to vaccine virus develops more promptly than the syphilitic reaction as judged from gross manifestations although it is known that changes do occur at the site of inoculation and probably elsewhere within a few days after the injection of *T. pallidum.*

The present results indicate, nevertheless, that vaccination did not bring about a state of "increased susceptibility" to the syphilitic infection, if one means by this term an increased liability to infection as indicated by a shortening of the incubation period of the primary lesion. But the findings amply demonstrate that it did markedly affect the syphilitic reaction, and since the effect was evident not only early but late in the disease, it is evident that the disorganization of the reaction was profound. The fact that it was evident for so long a time, as well as certain peculiar changes in the reaction such as the shortened time interval between successive phenomena, the marked increase in the number of secondary lesions and their general character suggest that the general alteration was of the nature of a decreased resistance on the part of the animals.

From knowledge already available, it is clear that the syphilitic reaction involves many factors that are essentially expressions of functional activity which, in some instances at least, appear to be related to changes in physical constitution. It is not unlikely that the differences brought out in the present experiments are the result of similar changes.

In conclusion, it may be pointed out that the study of experimental coexistent or concomitant infections is of value as a means of investigating the general problem of susceptibility and resistance to disease. Hitherto, this study has been confined almost entirely to individual diseases.

SUMMARY.

Experiments are reported in which was studied the effect of a concomitant infection, vaccinia, upon the syphilitic reaction of rabbits. Vaccine virus was inoculated intracutaneously on the side of the body at the time of intratesticular inoculation with *Treponema pallidum*.

The results showed clearly that the vaccination caused a profound disturbance in the syphilitic reaction, the ensuing syphilis being extremely severe. From an analysis of various features of the reaction, it appeared that the factor of host resistance was primarily concerned in the effects observed.

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